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**ADVANCES IN ENZYMOLOGY**

**AND RELATED SUBJECTS OF  
BIOCHEMISTRY**

**Volume VII**

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# **ADVANCES IN ENZYMOLOGY**

***AND RELATED SUBJECTS OF BIOCHEMISTRY***

**Edited by F. F. NORD**  
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# PERMEABILITY AND ENZYME REACTIONS

By S. C. BROOKS, *Berkeley, California*

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## I. Introduction

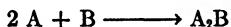
It has long been recognized that, in order to maintain metabolism, living cells must take in reactants and give off waste products, and that generally these activities are affected by the permeability of the plasma membranes of the cells. It is true that microscopically visible particles have been found to be taken up by or extruded through the cell surface in some cases, but such occurrences need not invalidate consideration of the permeability of the plasma membrane as a dominant factor in this relation. Here it appears that any attempt to trace the connection between permeability and enzymic reactions involves several aspects, namely: the relation between concentrations of reactants, etc., and rates of homogeneous reactions, between homogeneous reactions and rate of enzymic reactions, between rates of different steps in metabolism, especially rate of production of the measured end product; and finally the role of permeation, considered

to be parallel to reaction because of its effects on intracellular concentrations of substances involved in metabolism. The validity of the usual exponential equations for permeation must be examined in connection with the last topic. These will be applied to and exemplified by published experimental work.

## II. Theoretical Bases

### A. HOMOGENEOUS REACTION RATES AS AFFECTED BY CONCENTRATIONS OF REAGENTS

The law of mass action, proposed by Guldberg and Waage in papers in 1864, 1867, and 1879 (25), postulates that the rate of reaction is proportional to the product of the active masses (now thought of as activities) of reagent molecules. When two or more molecules participate in one reaction, each molecule enters independently, so that, *e. g.*, where the reaction reads:



the reaction rate is proportional to  $[A]^2 \times [B]$ , the brackets representing activities. This law is applicable to reversible reactions, which are to be treated as two opposed reactions in which the reactants of one become the products of the opposed reaction and vice versa.

To simplify the treatment, the difference between the thermodynamic active mass, *i. e.*, the activity, and the stoichiometric concentration will be disregarded here, and only the term "concentration" will be used. Actually quite wide differences between the two are encountered in dealing with such polar molecules as salts and proteins.

When the concentration of a reactant is by any means changed, the rate of reaction is correspondingly changed. If this reaction is one in which only one molecule of a reactant participates, the rate of reaction is proportional to the concentration of the reactant. This type of reaction is classed as unimolecular. Metabolic reactions are preponderately pseudo-unimolecular because of the great excess of one of the reactants, but they are also less frequently bimolecular. It is permissible to treat only those types of reaction in which a change in concentration of a reactant leads to a proportional change in rate of reaction. Some of the steps in metabolism liberate much free energy, so that the reaction appears to be irreversible. But many or most of the metabolic reactions are patently reversible, and it is important to note that the products of reaction in one sense become the reactants of the reverse reaction. Therefore anything increasing the concentration of a product will, by favoring the reverse reaction, appear to

oppose the first reaction. In a series of successive reactions, provided no irreversible steps intervene, this effect may extend beyond the reaction actually under consideration.

Furthermore, branched or complex chains of reactions can occur, so that, while an increase in the concentration of one initial reactant will accelerate all of the succeeding reactions more or less equally, a block in the removal of a product will divert the reaction to a parallel chain of reactions. Effects of this type make direct conclusions as to rate of reaction from the reagent concentrations open to careful scrutiny. The connection between cause and result in such systems is far from direct. Nevertheless, if we may disregard mutual effects of molecules on the properties of the system, it is permissible to accept the Guldberg-Waage law of mass action as a basis for studies of reaction, provided all the details of the reaction are known.

#### B. APPLICATION OF LAW OF MASS ACTION TO ENZYMIC REACTIONS

In the case of enzyme reactions there has been great lack of knowledge of the steps of reaction, so that some uncertainty as to the conclusions drawn even from *in vitro* reactions still persists. There is however an additional factor: dependence of the reaction on concentration and enzyme activity of the catalysts, and on concentration of the enzyme-substrate compound. The amount of enzyme may be taken as constant in any reaction mixture during the experiment. It is true that the contemporary view is that the enzyme (or prosthetic group or coenzyme) is changed by one reaction, and restored on completion of the reaction cycle. This would leave the enzyme concentration essentially constant, at least until the later stages of the reaction. The amount of the enzyme-substrate complex, the life of which may be brief or prolonged, depends on an equilibrium obedient to the law of mass action. With constant enzyme concentration, the amount of combined enzyme increases with concentration of substrate, and approaches constancy at high substrate concentration. Michaelis and Menten (55) express this relation in the equation:

$$K_M = \frac{[C_E][C_S]}{[C_X]}$$

where  $[C_E]$  and  $[C_X]$  represent the concentrations of free and combined enzyme,  $[C_S]$  is concentration of substrate, and  $K_M$ , the "Michaelis constant." This equation may be recast by calling the sum,  $C_X + C_E$ , the total enzyme,  $C_T$ , so that:

$$[C_X] = [C_T] \frac{[C_S]}{[K_M] + [C_S]}$$



This shows that the concentration of complex varies as the total enzyme concentration if  $[C_s]$  is kept constant, while, if  $[C_s]$  is varied, the last fraction accounts for the form of a saturation curve in the complex formation. Since it has been shown by Northrop (66) that the rate of an enzyme reaction varies as the enzyme concentration, the substrate concentration being kept constant, it is shown that the rate of reaction is proportional to the concentration of the enzyme-substrate complex. The Schütz law (79) proposed in 1885 has been shown by Northrop to be founded on experiments in which inhibitors are produced and lead to the incorrect concept that reaction rate is proportional to the square root of enzyme concentration.

It is apparent that, where the rate of a reaction is controlled by an enzyme complex, rate is proportional to substrate only approximately at very low concentrations; at higher concentrations rate becomes essentially independent of substrate.

Autocatalytic enzyme reactions are frequent, and in many or all of those known, the effect is due to production of an acid, such as phosphoric, lactic, pyruvic, or oleic acid. Here the rate of reaction changes with  $pH$ , since ionization and destruction of the enzyme at different  $pH$  values affect the amount of the active form of the enzyme. Hence no general rules can be set up, and it is not surprising that reaction rates do not follow changes in reactant concentration. For example, the degree of buffering of the reaction mixture will affect the  $pH$  changes produced by the autocatalytic acid, and hence the rate of reaction.

Inhibitors produced during the reaction may be protein compounds (66) which compete for the substrate and form unreactive complexes with it. Under suitable conditions it is possible to show that this competition obeys the law of mass action, but it is difficult to account for the relation between substrate concentration and reaction rate under less suitable experimental conditions.

The significance of this section depends largely on the fact that substrates and final products are generally easily diffusible materials, and enter or leave living cells while enzymes remain intracellular. Hence we are interested in the relation of permeability to enzyme reactions within the cells.

#### C. REACTION RATES IN A REACTION NET

The earlier formulations of Harcourt and Esson (27) and the formulations given by Mellor (51), Lewis (44), Osterhout (68), and others envision

relatively simple systems, particularly systems in which one reactant is converted by two successive irreversible unimolecular reactions through a second reactant into a final product. Proceeding from considerations of Nord and co-workers (64), a form more nearly useful for our purposes was developed (90,91). In this work Weichherz took into account relative volume of cells and suspending fluid, so that, in the case of relatively concentrated suspensions, it was important to take into account a diminution in the concentration of a reactant which was present in the suspending fluid and which had to enter the cell to enter into the reaction. The permeation into the cells involved a plasma membrane of definite surface and thickness, and the diffusion through this thickness is properly considered to obey kinetic equations like those of a reversible unimolecular reaction. A limitation not noted in the application of these equations was that they give the course of the first irreversible unimolecular reaction taking place in the cell, but that they are inapplicable to later reactions, such as carbon dioxide production (see page 16).

Figure 1 shows the types of curve for which Weichherz's equations were devised (90,91). In deriving these equations, still other limitations were noted. Where suspensions are so dilute that there is little perceptible decrease in the reactant which permeates the cells, the following equation holds:

$$-\frac{d\sigma}{dt} = \frac{\mu k v}{\mu + k} v C_0 (1 - e^{-(\mu + k)t})$$

Here  $C_0$  is the extracellular concentration of the reactant,  $\sigma$  its intracellular amount,  $v$  the volume of each cell,  $\nu$  number of cells, and  $k$  the constant of the reaction. The term  $\mu$  involves the area (page 8) and the thickness (page 8) of the cell membrane;  $t$  represents time.

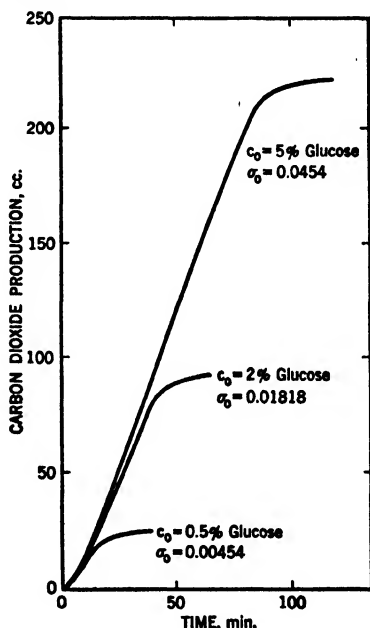


Fig. 1. Carbon dioxide produced by yeast in media of varying glucose content (90).

The form for concentrated suspensions is:

$$(-d\sigma/dt = \nu k C_0(e^{-\lambda_1 t} - e^{-\lambda_2 t}))$$

where

$$\lambda_1 = \lambda_2 = [2\mu + k \pm \sqrt{(2\mu + k)^2 - 8\nu\nu k}]/2$$

Weichherz selects values of these terms (some being experimentally fixed) and shows that these equations apply to curves of observed carbon dioxide production by yeast cells (Fig. 1).

Another approach to this general problem is given by Burton (7), who is, however, interested primarily in the idea of a "master reaction" first formulated by Blackman (2) and adopted by Pütter (70) and by Crozier (14). Burton points out that the use of equations for two successive irreversible reactions is increasingly misleading when the length of the series is increased and especially when some of the reactions are reversible. Although it is possible to accommodate to stipulated systems the equations given, the probability that they exist is so low that it is not profitable to develop this aspect. Addition of members in a chain of reactions



reduces the dependence of the later reactions on the reaction constants of earlier reactions. Note that this refers to reaction constants, rather than to the concentrations. However, if this deals with a steady state, the original reactant being kept at a constant concentration, then the presence of an irreversible reaction in this series introduces no change in the later reactions, except for a momentary disturbance when one of the earlier reactions suffers change in reaction rate. This is interesting in that, if one of these earlier reactions is permeation through a plasma membrane, then, if any one of many known influences changes the permeability, a situation of this type is usable. Burton (7) has also developed an equation for the concentration,  $x_M$ , of a member, M, in a long series,  $A \rightarrow B \rightarrow C \rightarrow D \rightarrow \dots M$ . In the equation, concentrations are  $x_1$ , the initial concentration of A, and  $x_M$ , that of the final product, M, at any time. Constants for the successive reactions are  $k_1, k_2, k_3 \dots k_M$ . The equation (for which the steps of development appear not to have been published) is:

$$X_M = X_1 \left\{ 1 - (-1)^{n-1} k_1 k_2 k_3 \dots k_{M-1} \sum_{j=1}^{j=M+1} \frac{e^{-k_j t}}{k_j (k_j - k_1) (k_j - k_2) \dots (k_j - k_{M-1})} \right\}$$

Burton has also discussed the steady state in reaction chains (8). Where it exists in a cell, and where source and sink are external to the cell

and are constant, the proportional amounts of the other reactants are determined by the rates of reaction. To the extent that any one or more reactions are determined by theoretical catalysts, the catalysts change the concentration but are powerless to change the flux of energy in the chain. However, enzymes appear to be, not theoretical catalysts, but rather reagents. The reactions must therefore be like bimolecular or higher-order reactions. Burton states that the equations can be used for orders higher than first, but it seems unavoidable that no accumulated reagent can produce a measurable increase in the rate of reaction occurring on the surface of a saturated enzyme. If the time required for the reaction itself to occur becomes larger than the interval in which a reactive position remains vacant, the usual rules fail. However, if the reaction proves to be free from these restrictions and if the values of source or sink concentrations are known, it is possible to calculate the concentrations from the reaction constants or vice versa. The equation developed is:

$$(k_0S - k_0x_1) = (kx_1' - kx_1'^2) = \dots = (k_zx_n - k_zZ) = R$$

Using various known parameters and derived equations like  $k_0S = R = k_0x$  it is possible to calculate values of constants or concentrations.

Here  $k$ ,  $k_0$ , and  $k_z$  are reaction constants;  $x_1$ ,  $x_2$ , and  $x_n$  are intermediate reactants; and  $S$  and  $Z$  are source and sink concentrations. In the same paper are calculated the costs of transition and temporary changes in concentration when one of the reaction constants has been altered. Since a change in the source concentration will have the same effect as a change in the corresponding reaction constants, the equations are applicable to the observed phenomena at the start of a reaction, for example, the phenomena found after introduction of glucose into a fermentation system. If  $y_1 = x_1 - \alpha$ ,  $y_1$  being the difference between the steady state concentration,  $x_1$ , and the momentary state concentration,  $\alpha$ , of the reactant,  $X$ , and if  $y_2 = x_2 - \beta$ , these values can be shown to be:

$$y_1 = ae^{-\lambda_1 t} + be^{-\lambda_2 t} \quad \text{and} \quad y_2 = ce^{-\lambda_1 t} + de^{-\lambda_2 t}$$

$\lambda_1$  and  $\lambda_2$  are the roots of the equation:

$$\lambda^2 + \lambda(k_0 + k + k' + k_2) + (k_1k_2 + k_0k' + kk_2) = 0$$

The values of  $a$ ,  $b$ ,  $c$ , and  $d$  can be equated to functions of  $\alpha$ ,  $\beta$ ,  $\lambda_1$ , and  $\lambda_2$ , and of the constants,  $k$ ,  $k_0$ , etc. From this,  $y_1$  and  $y_2$ , as functions of time, can be calculated. This equation gives the temporary difference between momentary and steady state concentrations, including the "overshooting," that is, a single oscillation of reactant concentration under specific conditions.

If the reactions involved may be considered to be free of the limitation imposed by the nature of enzyme action (as they might be even when enzymes are involved), these equations may prove useful. However Burton

warns that, in more complex reaction changes, the equations become very complex, and this is more important for reaction nets and reactions affected by the often crucial substrate-enzyme formation. One of the processes of metabolic reaction chains may be diffusion, for example, movement from one to another of the intracellular formed bodies which are associated with successive enzymes.

In general it may be said that Burton's treatment deals only with unbranched reaction chains, and it is obvious that branched chains in metabolism are probable. Their presence would complicate the picture. No treatment is practicable until more facts are known.

Gerard (24) has made a study of the influence of diffusion of oxygen in respiration by isolated cells—marine eggs like those of the sea urchin, *Arbacia punctulata*. He analyzed several possible cases, postulating as factors in his equations the diffusion coefficients, the oxygen consumption per unit volume, and the influence of spherical zonation, with different values of each of these. Only two such zones were considered, and, by postulating that the peripheral zone should be very thin and have a low diffusion coefficient, Gerard's picture would fit a plasma membrane and its permeability. Actually, if the thickness of the plasma membrane of a spherical egg of *Arbacia punctulata* ( $72\ \mu$  in diameter) is about  $200\ \text{\AA}$ , its thickness should be about 0.03% of the egg diameter. The permeability of the plasma membrane is not well known. The values given by Krogh (41) apply only to relatively large thicknesses of tissue ( $7.65$ – $290\ \mu$ ) in which nearly all of the permeation may well be regulated by relatively much thinner membranes, totaling perhaps  $0.05$ – $0.5\ \mu$ . Krogh's permeability to oxygen was 0.14 for muscle and 0.115 for connective tissue in terms of cubic centimeters of oxygen traversing  $1\ \text{cm.}^2\ \text{min.}^{-1}$  under a gradient of 1 atm. across a layer of  $1\ \mu$ . If this is referred to the thinner plasma membranes, supposing them to be about  $1/300$  of the total thickness, the permeabilities become 0.00047 and 0.00038, respectively. The permeability to water remains 0.34, as Krogh gives it, and the ratio of permeabilities becomes about 800.

In developing his equations Gerard has used widely different values. It might be thought that these equations should be fitted to these conditions, but there is also the difficulty that the rate of oxygen consumption of any region is assumed to be independent of the concentration of oxygen. This presumes the essential saturation of some enzymes such that only when oxygen concentration is extremely low does the amount of this complex begin to diminish apparently. The lack of information as to this

limit makes it difficult to apply these equations to the effect of permeability on enzyme action. It should be made clear that Gerard's goal is to account for the relation of the oxygen consumption of eggs to the oxygen concentration in the circumambient system, the whole system being in a steady state. This is aside from the present uses, and no further mention will be made of this work. Here it is qualitatively apparent that permeation of oxygen and removal of carbon dioxide and other wastes will control the relative concentrations of the intermediates, which are imperfectly known. Burton's paper (8) on steady states is useful in this connection.

#### D. DIFFUSION AND PERMEATION AS EXPONENTIAL PROCESSES

It has been tacitly assumed that permeation and, more generally, diffusion into closed volumes like living cells lead to an increase in concentration of the permeant within the cell which follows an exponential time curve. On this basis permeation may be treated mathematically as a unimolecular reaction. Since permeation reaches an equilibrium when the external ( $C_E$ ) and internal ( $C_i$ ) concentrations are equal, this equivalent unimolecular reaction must be reversible, with  $K = k_1/k_2 = 1$ . This of course neglects changes in activity coefficients of the permeant.

The fundamental equations of Fick (22) describe the rate of unidimensional diffusion of material moving along an infinite system. It is important to examine the validity of the assumption that these equations describe to a satisfactory degree the situation in quite different systems. For example, the experimental evidence available in biology concerns permeation into spherical cells or cylinders, or cells with more complicated shapes, like the frequently observed mammalian erythrocytes.

The Fick equations deal with the statistical result of movement of large numbers of molecules, and these are satisfactory for the present purpose. The equation is:

$$dn = Da (dc/dx)_i dt$$

for the rate of diffusion, and, for rate of change of concentration, the equation is:

$$(dc/dt)_x = D(d^2c/dx^2)_i$$

Here  $dn$  is the amount of substance which passes across an area,  $a$ , in an ideal plane transverse to the diffusion in time,  $dt$ , where the concentration gradient is  $dc/dx$ .  $D$  is the diffusion constant of the reactant. For use, the latter equation, often termed the fundamental equation, must be integrated. Stefan's (81) solution modified by Svedberg (83) applies to diffusion of a solute in a vessel of uniform cross section from an infinitely long column of solution into a similar column of solute. This treatment follows that

given by Hitchcock (32). Where  $y = (x/2)\sqrt{Dt}$ , this equation states that concentration,  $c$ , is:

$$c = \left(\frac{c_0}{2}\right) \left(1 - \left(\frac{2}{\sqrt{\pi}}\right) \int_0^y e^{-y^2} dy\right)$$

illustrating the exponential dependence of concentration on time and justifying application to this idealized system of the same isotherm as that for unimolecular reactions.

Is this dependence valid for other systems? In the first place, permeation into cells is ordinarily considered to take place from a relatively large volume of well-stirred medium, so that  $c_0$  remains essentially constant. Therefore the  $c_0/2$  of the equation above becomes  $c_0$ . Any deviation from these conditions merits special treatment.

Spherical and cylindrical cells are of interest. If there is no plasma membrane with low permeability and diffusion is pictured as taking place in a homogeneous cylinder, the rate of entrance of permeant is given by:

$$\frac{dx}{dt} = \frac{D}{r} \frac{d}{dr} \left( r \frac{dc}{dr} \right)$$

instead of by the equation for unidimensional diffusion:

$$\frac{dc}{dt} = D \frac{d^2c}{dx^2}$$

Here  $r$  is the radius of the cylinder into which permeation, to keep the study simple, takes place only through the cylindrical surface, and not through the ends. The time course of mean intracellular concentration can be plotted from the derived equations, for which consult Hill (31) or Jacobs (36) who gives an equivalent form of the equation. See also Rashevsky (72).

It is apparent that the time course of the average concentration within the cylinder is affected by reflection, and is no longer an exponential curve. Hill has illustrated the operation of this equation in the case of nerves or muscles, *in vitro* (therefore lacking circulation). In the same way that Krogh has dealt with tissue, assumed homogeneous, Hill has used the equation justifiably for such cases. However, this is not applicable to isolated cylindrical cells like fungi, nor to many algal cells. Collander and Bärlund (12) have shown the difference between this equation for diffusion into cylinders and that for diffusion through a relatively impermeable membrane, using the alga, *Chara ceratophylla* (Fig. 2). This cylindrical cell contains a large vacuole (diameter up to 1.8 mm.) surrounded by a thin layer of protoplasm (thickness about  $5 \mu$ ), and, although it is considered that an outer and an inner plasma membrane, still thinner, actually regulates permeation, it is permissible to test the equations by considering the protoplasm as a whole as a permeable membrane. Here the data show

that permeation is dominated by the relatively impermeable protoplasmic layer, and consequently that the time course of the vacuolar concentration of the permeant, essentially uniform, is exponential. The concentration obeys the equation:

$$c_1 = c_0 (1 - e^{-Dt}),$$

Similar situations seem to typify isolated cylindrical living cells, which regulate their permeation by their plasma membranes.

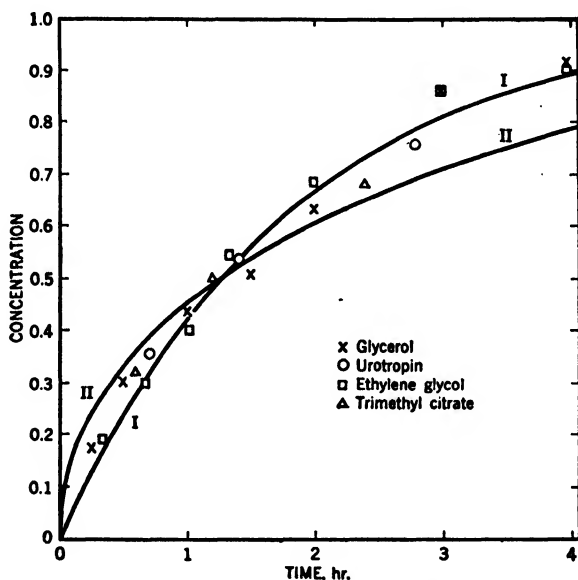


Fig. 2. Penetration of four solutes into cells of *Chara ceratophylla* (12). The ordinate is a scale in which 1.0 represents equality of concentrations of solutes inside and outside the cells. Curve I, diffusion through a thin layer; curve II, diffusion into a homogeneous cylinder.

For spherical cells, like yeast or marine eggs, similar considerations apply. The corresponding equation for homogeneous spheres is:

$$\frac{dc}{dt} = \frac{D}{r^2} \left( \frac{d}{dr} r^2 \frac{dc}{dr} \right)$$

which does not lead to an exponential time concentration. Here again in practice one can derive equations, assuming the relatively thin plasma membrane as an essentially plane layer, so that exponential equations again be-



come applicable to these processes. All the equations given here can be altered for use when the permeant is combined by a metabolic reaction or otherwise, and thus is removed from its diffusion field. This was done by Gerard (24) (see page 8), Hill (31), and others.

#### E. STRUCTURE OF THE PLASMA MEMBRANE AND OTHER SURFACES

Assuming the validity of the Fick laws and the modified equations derived from those given above, there still remains the question of the role of the plasma membrane of living cells. Davson and Danielli (15) state that, "unfortunately, Fick's equation is not necessarily applicable to such thin layers," *i.e.*, the plasma membrane. Danielli here postulates that this membrane is a homogeneous layer of lipides about  $10^{-8}$  cm. thick, with surface layers of adsorbed proteins. This position seems untenable in this extreme form, although the underlying picture of one or more bimolecular layers of lipid molecules, the hydrocarbon chains perpendicular to the cell surface, has found much support. This type of micelle appears parallel to the surface of sodium oleate hydrogels (85). It appears in cell surfaces (20,21,78), in nerve axon sheaths (77), and in formed bodies within cells (58,76). In the more recent work (76,78) the authors speak of micelles of lipides rather than of continuous layers. These micelles appear to be enmeshed in protein films lying more or less parallel to the surface. Thus Schmitt and Bear (77) state that "radially oriented lipid micelles, presumably interspersed between concentric layers of protein . . . material form the basis of the organization" of vertebrate nerve axon sheaths. The thesis of Danielli and Davson that the plasma membrane has a continuous or homogeneous sheath appears to depend on failure to consider all the evidence available, so that probably the plasma membrane typically contains a framework of protein molecules in which lipid micelles are caught, usually parallel to the surface so that the hydrocarbon chains are perpendicular to it (see Brooks, 6). Such a structure may logically be derived by a loss of the smaller solutes from between proteins on exposed surfaces of protoplasm. Protein molecules are elongated; molecules like fibrin have been reported by Cohn *et al.* (10) to have an axial ratio of about 30, and appear to be capable of intertangling. This reaction has been likened to blood coagulation (29) in that reaction occurs between protein molecules holding them together. This hypothesis presents interesting possibilities.

Danielli (15) shows that the permeation of molecules through a lipid layer requires a minimum kinetic energy for passage past energy barriers, of which those for transition between water and oil phases are prominent.

This idea is adaptable to any type of membrane, regardless of the supposed unavoidable passage through oils.

Proteins obviously retain molecules by various types of force at different positions, and in general it must be recognized that this idea is valid. Danielli suggests a modification of his formula which must be used if the plasma membrane is not a "homogeneous" lipid layer, but obviously the modification is not usable because of lack of data. However, even if this membrane is of Danielli's type, he concludes that the passage of a solute obeys the rule:

$$\frac{ds}{dt} = \frac{as}{nb + 2e} (c - c_1)$$

where  $s$  is quantity of permeant,  $c$  and  $c_1$ , concentrations of permeant on the two faces of the membrane,  $n$ , number of maxima of a certain required kinetic energy, and  $a$ ,  $b$ , and  $e$ , constants. The right-hand fraction reduces, for any given case, to the permeability constant,  $P$ , and we come back to the first Fick equation.

Therefore there is no reason, in any of the cases cited, for attributing any properties to the plasma membrane which invalidates the use of the diffusion laws developed above.

### III. Experimental Work Relating Enzyme Reactions to Permeation of Reactants

#### A. PERMEATION OF GLUCOSE

A great deal of attention has been directed to the anaerobic fermentation by yeasts, notably *Saccharomyces cerevisiae* in its various forms (top and bottom brewers' and bakers' yeast). This has been industrially important, and the subspherical cells, about 6-8  $\mu$  in diameter, are produced under standard conditions. They can be brought into suspension with little or no clumping. They are then suitable for tests of the permeation through the surface of the suspended cells. From the discussion on pages 9-13, it follows that permeation can be treated either as the diffusion into spheres, where there is no semipermeable plasma membrane, or as the unidimensional diffusion through a relatively thin, slightly permeable membrane, with substantial complete diffusion of permeant. Since there is every reason to assume the latter case, the former case is considered unimportant.

Slator and Sand (80), however, adopted the latter idea, *i.e.*, there is no plasma membrane. A similar neglect of the plasma membrane occurs in the work of Krogh (41) and Gerard (24) noted on page 8. Slator and Sand concluded that the diffusion of glucose is about like that of mannitol

in water (about  $5-6 \times 10^{-6}$  cm.<sup>2</sup> sec.<sup>-1</sup>) and concluded that glucose would reach the site of its utilization in ample quantity. (See also Sand, 75, who studied the diffusion of reactants to the surface of colloidal catalysts.) They used a consumption of glucose of about  $3 \times 10^{-14}$  gm. sec.<sup>-1</sup>. The supply was calculated to exceed this use by some  $10^5$  to  $10^6$  times.

But, if the permeability of the yeast cells is calculated on the assumption that the actual supply is of the same magnitude as the observed use, one obtains a permeability constant of  $7.0 \times 10^{-10}$  cm. sec.<sup>-1</sup>. In reaching this result, the values for the area were taken as  $1.65 \times 10^{-6}$  cm.<sup>2</sup>, and the concentration decrement across the membrane may be taken as about one half the 5% glucose medium, i.e., 0.2 M. Naturally this value might be significant only, at most, to the degree of magnitude. However, one may note the cited permeabilities to glucose are not far from this order of magnitude:  $0.8 \times 10^{-11}$  for the external and vacuolar membranes in succession of the alga, *Chara ceratophylla* (12); about  $5 \times 10^{-9}$  for human erythrocytes, while much lower for other laboratory animal erythrocytes (87); and probably about  $10^{-11}$  for eggs of the marine annelid *Chaetopterus* (46), for which a value of  $1.05 \times 10^{-9}$  was reported for permeability to glycerol.

Therefore the permeability of yeast seems more probably to be of the order requisite for allowing glucose to penetrate from about 5% solution, about as fast as it is being used. This situation is obviously a case of steady state, and it is apparent that all processes, including the liberation of carbon dioxide usually used as a measure of metabolism, are adjusted to the rate of permeation of glucose. The question of whether higher permeability would increase the rate of carbon dioxide production or metabolism generally, or would be limited to a "maximum rate" by a mechanism such as that of the Michaelis-Menten concept will be treated on page 18.

Another effect by which permeability shows itself is evident during the beginning of fermentation, i.e., when yeast which has used up its store of carbohydrates is suspended in a glucose solution and measurements are begun forthwith. If permeability to glucose were very low, it is obvious that at first very little carbon dioxide would be produced, while later on it would be produced at a "maximum rate." This situation is one of the "disturbances" in steady states discussed by Burton (8) (see page 7). But Burton's treatment is usable only for the simple case,  $S \rightarrow A \rightarrow B \rightarrow Z$ , in which the "cost of transition" can be calculated. Longer and more complex chains require very complex calculations. No such solution has been offered. More important is a solution of the steps between permeation of glucose and the liberation of carbon dioxide from the medium.

In fermentation no oxygen is used, so that there is no question as to permeability to oxygen. Glucose, provided in the medium, must permeate the yeast cell before metabolism starts. Metabolism, probably by means of several steps leads to the liberation of carbon dioxide presumably by decarboxylation. To be measured, this carbon dioxide must pass out through the plasma membrane and be freed as a gas from the medium (see Nord and Weichherz, 64). The very great permeability to carbon dioxide of all or most of all the studied types of plasma membrane leads to the conclusion that this step has no measurable influence. The liberation of carbon dioxide from even saturated solutions has been thought to require the use of special methods, such as the addition of large amounts of citric acid as Meyerhof advocates (53). Further study of this step is desirable.

The intermediate steps of metabolism have been the subject of much discussion. Some aspects will be treated briefly below. Meyerhof (54) states that the glucose molecule "passes anaerobically through twelve stable intermediary steps before forming alcohol and carbon dioxide; at least three dissociable organic enzymes, twenty or more enzyme proteins and some bivalent metals (Mn and Mg) are necessary for the breakdown." This is a widely accepted conclusion from the work on fermentation by yeast extracts (press juice, maceration juice prepared from dried yeast, frozen yeast, or mechanically disrupted yeast) in which phosphorylation is considered important in desmolysis. Decarboxylation of pyruvic acid appears to be the source of the carbon dioxide. Obviously then, carbon dioxide production is far from the initial step of permeation of glucose.

Although it is not the province of this paper to discuss in further detail the presence or absence of phosphorylative desmolysis within living yeast cells, it is still necessary to discuss the discrepancy between fermentation by cells and that by extracts, since several pathways are possible.

It has long been known that fermentation by yeast extracts normally comprises at least three phases: an initial induction period followed, within a matter of about five or ten minutes, by a very rapid fermentation; this persists for some time and passes over into a prolonged relatively slow action. All this has been measured by carbon dioxide production. This is true not only for glucose, shown, *e.g.*, by Meyerhof (53,54), but also for galactose (40) and for fructose (34). These have been attributed to the formation of the hexose-6-phosphate (Robison ester) which must precede any carbon dioxide liberation, and the conversion of this to hexose-1,6-diphosphate (Harden-Young ester), which ferments slowly. The maximum of hexose-6-phosphate concentration coincides with the maximum car-

bon dioxide production. The several supposed steps in desmolysis which intervene between the ester formation and decarboxylation have been widely discussed.

The most conspicuous method of desmolysis seems to be that of splitting the 1,6-diphosphate into two phosphorylated three-carbon compounds. Nevertheless other pathways are known. The oxidation of hexose-6-phosphate through the corresponding hexonic acid has been suggested by Warburg, Christian, and Griese (88,89). The hexose diphosphate or the fructose-6-monophosphate (Neuberg ester) were not easily attacked. The first product, phosphogluconic acid, can be fermented by yeast maceration juice (Lebedev juice) yielding carbon dioxide. Lipmann (45) consid-

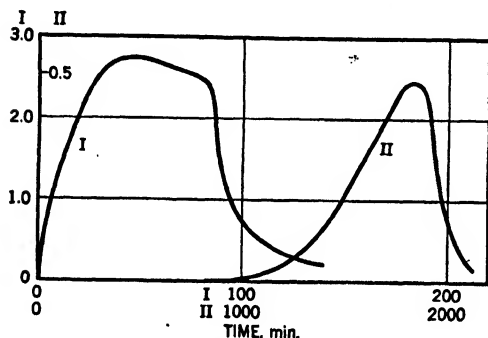


Fig. 3. Carbon dioxide produced by living cells (I) and by a cell-free yeast extract (II) (92).

ers that the formation of phosphohexonic acid was the first step of an independent chain of oxidative metabolism, to be followed by oxidation to the corresponding  $\alpha$ -keto acid and its decarboxylation, thus yielding a five-carbon sugar. Dickens (16,17) has studied this cycle with oxidative decarboxylation to the lower acid. He traces this process from the phosphorylated sugar, through five-, four-, three-, and two-carbon compounds, liberating carbon dioxide in successive steps. Although his work was based on animal material, it is still conceivable that at least two branches of oxidation are possible in yeast, *viz.*, the direct formation of three-carbon compounds from the 1,6-diphosphohexoses, and the stepwise decarboxylation, either oxidative or nonoxidative. Breusch (3,4) emphasizes these two principal systems of respiration of mammalian tissues.

In any case, even the liberation of carbon dioxide by yeast is not a direct consequence of glucose permeation, but is a result of a branched chain of metabolism, in which not less than four steps are necessary.

The three phases just discussed, or at least the first two, were noted by Nord and Franke (63). Weichherz and Nord (92) found a difference between the time curves of carbon dioxide production for cell-free extract and living yeast cell fermentation, especially during the induction period.

They illustrate the difference (Fig. 3); no other data are given. Meyerhof (53), however, gives data for cell-free fermentation like that given by Nord, but for the first ten minutes after glucose addition, while the curves of Nord give no detail for such short periods. In other words it cannot be said that there is any definite evidence that there is a difference between the two types of metabolism. Meyerhof (54) has recently found that most yeast juices lack adenosine triphosphatase ("apyrase"), which is bound to the cell structure and is sensitive to drying, etc. If it is conserved by using methods like supersonic cell fragmentation, or is added from foreign sources, no difference has been found by him between the fermentation by the juice and that by living yeast, provided the yeast is kept well in suspension. This situation was worked on by Lynen (47) and by Nilsson and co-workers (59-62), but the most recent work was that by Meyerhof. This theory concerns the dephosphorylation of 1,3-diphosphoglyceric acid, allowing its rapid fermentation. Therefore the induction period is not involved.

Nord (64) observed that the induction period is greatly decreased by adequate stirring of their 3-10% yeast suspension. There are also effects of stirring considered by Weichherz and Nord (92) in the latter part of the fermentation, *i.e.*, a phase corresponding to the slow fermentation phase noted by Meyerhof. They conclude substantially that a phosphorylation-free breakdown of substrate molecules must be considered to occur parallel to the dissimilation of phosphorylated substrates in living yeast cells. While there seems to be little tendency now to consider that phosphorylation-free breakdown may occur in yeast, yet it seems quite possible that several different reaction chains occur, and two or three pathways have been shown to occur in systems derived from yeast. This situation may well refer to these different mechanisms of desmolysis.

If this is true, the question arises as to the significance of Nord's interpretation of the effect of stirring, namely, that the *remaining* induction period, when stirring is adequate, is due to the relatively slow permeation of glucose into the yeast cells, followed by the freeing of carbon dioxide. Weichherz (90,91) has derived equations on these assumptions, obtaining among others the one given also in the treatise of Nord and Weidenhagen (65):

$$-\frac{d\sigma}{dt} = \frac{\mu k}{k - \mu} s_0 (e^{-kt} - e^{-\mu t})$$

which is a solution of:

$$\frac{d^2c}{dt^2} + (\mu + k) \frac{dc}{dt} + \mu k c = 0$$

Here  $\alpha$  is the amount of glucose in the medium,  $c$  its intracellular concentration,  $k$  the unimolecular reaction constant, and

$$\mu = \frac{f\alpha D}{v\delta} \frac{V_0}{2V + v f \delta}$$

where  $f$  is the total surface and  $v$  is the volume of each of the yeast cells in the system.  $D$  is the diffusion coefficient,  $\alpha$  a coefficient expressing the relation between permeation through the plasma membrane and its free diffusion in extra- or intracellular media,  $\delta$  the thickness of the plasma membrane, and  $V_0$  and  $V$  are the volumes of the total and of the extracellular mediums. Other notation is customary.

This equation gives the reaction rate,  $ds/dt$ ; this was integrated and the observed fermentation was found by Weichherz (91) and Nord and Weichherz (64) to obey the integration. It was concluded that the first constant,  $\mu$ , is one proportional, under otherwise equivalent conditions, to permeability.

From the values for  $-(\partial\sigma^2/\partial t^2)_{t=0} = 2\mu\nu vk\sigma_0$ , where  $\nu$  = number of yeast cells (so that  $\nu\nu$  = total cell volume), which can be converted to  $(f\alpha D/\delta V)\nu k\sigma_0$ , it is possible to calculate from the observed values the value of  $\alpha k/\delta$ , other values being known. This was derived by Weichherz (90) and corrected in a later paper (91). In the latter a value for  $\alpha k/\delta$  of  $0.366 \times 10^{-8}$  was given. Using an approximation given in the earlier paper which assumes  $\delta$ , the thickness of the plasma membrane, to be between  $8 \times 10^{-6}$  and  $6 \times 10^{-6}$  cm., and  $k$ , the unimolecular reaction rate constant, to have a value between 0.1 and 1.0 mole min.<sup>-1</sup>, one obtains a mean value of  $\alpha = 1.32 \times 10^{-11}$  cm.<sup>2</sup> min.<sup>-1</sup> or a minimum of about  $0.2 \times 10^{-11}$ . The permeability,  $P$ , is equal to  $\alpha D$ , where  $D$  is given as  $2.756 \times 10^{-4}$ , or  $P = 0.551 \times 10^{-15}$  cm.<sup>2</sup> min.<sup>-1</sup>. The most recent determinations indicate a value for  $D$  of  $3.6 \times 10^{-4}$ , but the difference is immaterial in this connection (see Hitchcock, 32).  $P$  depends on two estimates, the membrane thickness and a unimolecular reaction constant, for which little or no evidence can be adduced.

The permeability to glucose of a different plant, the alga, *Chara ceratophylla*, can be estimated from data in a paper by Collander and Bärlund (12). It should be about  $2.5 \times 10^{-9}$  cm.<sup>2</sup> min.<sup>-1</sup> if one assumes that the thicknesses of the exterior and vacuolar membranes comprise  $5 \times 10^{-6}$  cm. This value, which is the maximum possible quantity, is about a million times higher than that derived from the values assigned by Weichherz. This difference may be thought to be due to the possibly much less permeable plasma membrane of the yeast or to an unfortunate value of the second constant, that of a unimolecular reaction rate constant. From this point of view it is quite apparent that an estimate of the permeability of yeast cells to glucose would be of interest. An estimate has been calculated on page 14 and found to be approximately  $7.0 \times 10^{-10}$ . Another approximation can be obtained from data given for the steady state by Medvedev

(50), whose mathematical equations relate to adsorption on the cell surface and seem not to be useful. His figure for the rate of breakdown of glucose is  $0.3 \times 10^{-15}$  mole sec. $^{-1}$  cell $^{-1}$  in 5% glucose (= 0.417 mole). The same amount must pass through the plasma membrane. Assuming that half of the whole concentration acts across the membrane and that the dimensions given by Weichherz are valid, the permeability is found to be  $5 \times 10^{-10}$  cm. $^2$  sec. $^{-1}$ . This is substantially the same as the other permeability constant given on page 14.

If the next step and all others up to the first irreversible step are rapid, higher values for permeability will be found, that is, the same rate of permeation obtains with a lower concentration gradient. This leaves Weichherz's very low figure of permeability lacking corroboration. If the value for  $k$  in  $\alpha k/\delta = \text{constant}$  were to be greatly lowered, the permeability, proportional to  $\alpha D$ , would be brought into accordance with admittedly weak but still preferable estimates of permeability.

However, if it is admitted in the beginning that fermentation is a complex process, and that other factors than permeability are the rate-determining factors in this process, the whole problem is relegated to the domain of the unsolved. It might be better to admit that so far we have no evidence of any detectable effect of permeability on the shape of the time curve for fermentation in the induction phase.

It has been argued with justice that high glucose concentrations,  $> 0.5 M$ , withdraw water from the living cell including its plasma membrane, thus lowering the permeability of the latter. This has been urged by Nord and Weichherz (64) in connection with the changes in fermentation rate in glucose solutions of different concentrations, 0.25 to 20%. There was earlier work by Slator and Sand (80) which seemed to the above authors to be discrepant. The more recent work of Hopkins and Roberts (33) explains the discrepancy on the basis of the Michaelis-Menten concept. Here again it was found that stirring must be adequate for the experiment to yield significant data. Taking into account that the loss of glucose into the cells is a factor in the more concentrated suspensions, *e.g.*, 10%, where the distance between cells is less than the cell diameter, these workers found that at low yeast concentrations the Michaelis-Menten concept is important. Here the rate of fermentation,  $r$ , is related to maximum rate,  $R$ , of fermentation when the enzyme is saturated with the substrate, glucose, by the relation:

$$\frac{1}{r} = \frac{K_M}{Rr} + \frac{1}{R}$$



where  $x$  is glucose concentration and  $K_M$  is a constant. This leads to different forms according to the relative concentration of yeast and of glucose. These are given, and are found to hold. Although there may be concentration effects present, they are not evident in the data.

It must be noted that these authors also assume that fermentation is a unimolecular reaction. It has been repeatedly stressed here that quantitative evaluation is not possible unless the constants and rates of diffusion are known. Nevertheless the general sigmoid form for the concentration curve of the final product, here measured, is known; and Burton's equation (see page 6) may be used for it.

This treatment is obviously limited by unknown factors, including the probable reversibility of many or all of the reactions, as well as the reaction with reagents present in finite amounts in the cell, such as enzymes. Probably therefore the Weichherz and like equations developed for two-step processes are only fortuitously similar in their prediction to those actually observed during fermentation, where much longer and more complex reaction systems are present. Such equations lack quantitative significance. Probably stirring is important in that it maintains a concentration of a reagent on the surface about like that in the bulk of the medium. But equations fail to tell us about permeability, or about its possible significant effect on the enzyme reactions.

## B. PERMEATION OF NARCOTICS

It is recognized that narcotics reduce the rate of metabolic, presumably enzymic, reactions. This refers to a steady state, but it is not yet feasible to speak with any confidence of the role of cellular permeability to reactants or products. The narcotics in many cases appear to alter the structure of plasma membranes and increase or decrease their permeability. But the picture is far from clear. It seems needful to keep in mind the alternative possibility of competitive combination of the narcotic with an enzyme or enzymes involved in the metabolic reaction studied. It is now recognized that the differences between so-called primary and secondary valences are expressions of the combined attractions and repulsions caused by electrons and nuclei and can be described as ionic forces, or as permanent or transitionally induced dipole forces and their orientation and position. This will not be discussed here further; the reader is referred to Bateman (1a) and Mark (49). It will be recognized that "adsorption" is a concentration of the adsorbate in an energy trough around the adsorbing group. It is a reaction in the full sense.

Direct determinations of the adsorption of ethylene on egg albumin, urease, sodium oleate, and other sols have been made by Nord and his associates (91a). These show that only in the case of the sodium oleate is the adsorption conspicuous, although they do not completely exclude adsorption on egg albumin, or on untested cellular enzymes. The indirect evidence from the increase in surface tension and the decrease in viscosity of colloids produced by ethylene suggests that adsorption occurs. But ethylene is considered not to show resonance (69a), a prerequisite for the presence of the polarized form suggested by Nord and Franke (63a, 63b) as the basis for its adsorption. It seems still undecided whether or not ethylene is adsorbed at strategic positions on any of the cellular fermentation enzymes. The increased carbon dioxide production in the presence of an ethylene film is well established by Nord *et al.* (62a, 62b, 63, 63a, 63b) but there is less evidence as to the manner of action of ethylene.

Studies by Nord and Franke (63, 63a, 63b) raise the question of the role of permeability in the increase by ethylene of the rate of fermentative carbon dioxide production by living bottom yeast during the first approximately 50 to 80 minutes after exposure to bubbling ethylene and addition of glucose to the system. This increase passes into a decrease, a change attributed to "protective" adsorption of the ethylene onto the enzyme. There has been much doubt as to the effect of narcotics on permeability, and it appears that there is no general rule. It had been suspected by Osterhout (67) for a marine alga, by Guttman (26) for frog sartorius muscle, and for squid giant axons by Cole and Curtis (11) that low concentrations decrease permeability. On the other hand, the permeability of some erythrocytes to glycerol is decreased by *n*-butyl alcohol while with other erythrocytes the change is opposite, as found by Jacobs and Parpart (37). Bär-lund (1) shows that ether in a suitable concentration increases the permeability of the brackish alga, *Chara*, to ethylene glycol, urea, etc., and decreases the rate of intake of the lithium ion.

The manner of action of narcotics has been discussed extensively, and some of these discussions are relevant to permeability. The adsorption theory of Traube (86) has not proved to be successful in many cases. It seems questionable for ethylene, for reasons just discussed, and probably for the action of ethylene on permeability. The "lipoid theory" of narcotics has been advanced by Overton (69) and Meyer (52), and seems stronger. It depends on the correlation between narcotic effect and the distribution coefficients between lipides and water. Those narcotics with higher solubilities in lipides have been widely found to have higher narcotic effects.

This is important for permeability, since plasma membranes appear to contain a high proportion of lipides, as shown, *e.g.*, for erythrocytes by Dziemian (18). Ethylene would be able, by virtue of its apolar structure, to dissolve in the lipide phase and to counteract the bonding effect of the double bonds of the lipide hydrocarbon chains. At first, however, the presence of additional apolar molecules may account for the frequently observed decrease in permeability, while the ultimate invasion of the membrane by ethylene would disrupt it, with increased permeability. This permeability is here thought of as applying to glucose and other molecules highly soluble in water.

This terminal <sup>slow</sup>increase in permeability has often been found to be irreversible, and this irreversibility is generally acknowledged. The increase noted by Nord (62a,62b), and Nord and Franke (63,63a,63b) may form an exception, in that this effect disappears after 30–60 minutes or more. Alternatively it may be concluded that the increase is quasi-permanent, while inhibition of the cell enzymes supervenes, thus concealing the effects of permeability changes. The inhibition by ethylene of the zymase activity of cell-free yeast extracts shows that decrease in cellular permeability is at most of only minor importance.

Fisher and Stern (23) studied the effects of urethan (ethyl carbamate) on the oxygen consumption by yeast. The medium contained 5% glucose. They plotted the logarithm of the ratio of uninhibited respiration to inhibited respiration against the logarithm of concentration of narcotic, and found the relation shown in Figure 4. In the figure is found a linear relation between the two terms given above (here called  $x$  and  $y$ , respectively), the slopes being given by term  $a$  in  $\log x = a \log y$ . This is thought to signify that a number,  $a$ , of molecules of urethan combine with one enzyme molecule. From the observed occurrence of two distinct slopes it was concluded that the urethan had combined at the higher concentrations with an enzyme (possibly a flavoprotein, if one may reason as has been done for Chloretone, 56), but at lower concentrations with another enzyme concerned with "activity" (growth?) of the yeast.

The question might be raised whether the first slope was an expression of a reduction of permeability to oxygen by this narcotic. The relatively low permeability to oxygen was shown by Hartridge and Roughton (28), who carried out experiments on the combination of oxygen with hemoglobin in intact red blood cells and in laked blood, where this process occurs much faster. Interference with the oxygen supply might retard its consumption. For this effect a relatively large number of molecules of ure-

than, per molecule of some ingredient of the plasma membrane (shown by the slope of the curve), would be needed. It is frequently recognized that only extremely low oxygen pressures affect respiration. Shoup is quoted by Taylor (84) as finding that an oxygen pressure of only 2 mm. was able to depress respiration of luminescent bacteria to 50%. Experiments on the strain of luminescent bacterium used by Taylor (84), probably *Photobacterium fischeri* (*Achromobacter fischeri*) or *Achromobacter harveyi*, showed

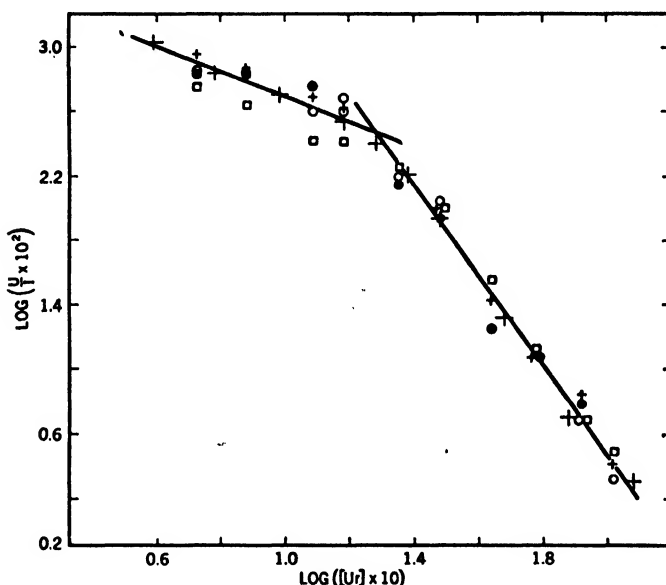


Fig. 4. Effect of urethan on oxygen consumption by yeast (23). The ordinate is the logarithm of the ratio of the uninhibited to the inhibited oxygen consumption. Three runs are represented by different symbols; large crosses are theoretical points.

that luminescence and respiration both exhibit a response increased at low concentrations of urethan and decreased at higher concentrations. But the luminescence, apparently a correlative of the "activity" of Fisher and Stern, is more sensitive to urethan than the oxygen consumption is. This initial stimulation and the subsequent inhibition are difficult to envision as an effect on permeability. The luminescence appears to be a reaction of a substrate (luciferin, a reduced alloxazine compound) which gives its hydrogen to oxygen in the presence of luciferase, which takes up the energy of reaction and spontaneously liberates it as light. See McElroy (48) and

Johnson, Eyring, and Williams (38), as well as the chapter by Johnson in this volume, for a discussion of this subject. Oxygen consumption is therefore directly connected with luminescence, and permeability might produce effects on luminescence as well as on oxygen consumption without the necessity of devoting attention to intermediate steps. On the basis of this work one is led to consider that permeability to oxygen may be significant in the experiments of Fisher and Stern.

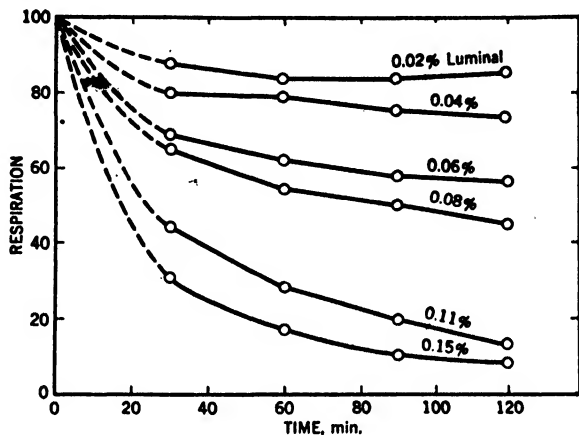


Fig. 5. Oxygen consumption by slices of rat brain cortex in the presence of Luminal (control respiration equals 100) (39).

A possible parallel case concerns the effects of Luminal on oxygen consumption by slices of rat cortex. Jowett (39) has measured  $Q_{O_2}$  during successive 30-minute periods for Luminal concentrations, and finds progressive decreases in  $Q_{O_2}$  values during 60 to 120 minutes. It may be noted (Fig. 5) that plotting total oxygen consumption during the two hours yields a broken curve apparently like the curves obtained by Fisher and Stern (23). It is evident that the penetration of Luminal into the tissues occurs, but the precise part taken by the permeability of the cells as opposed to intercellular diffusion is not clear. Again it can be pointed out that, if Luminal affects flavoproteins (56), only two reactions precede the inhibited reaction: cytochrome oxidase oxidation by oxygen, and its reduction by the flavoprotein. Cytochrome reactions seem to be involved also, but not in the direct line. Since we are dealing with reversible reactions which are very rapid, it is quite understandable that the narcotic effects

are directly reproduced in oxygen consumption. Therefore it is not improbable that permeability is a real factor here also.

### C. PERMEATION OF MERCURIC CHLORIDE

Although the work on luminescent forms gives no positive evidence of permeability as a factor in oxygen consumption, Houck (35) found effects of mercuric chloride ( $\text{HgCl}_2$ ) on the luminescence of *Photobacterium fischeri* which may be referable to permeation. It requires approximately 160

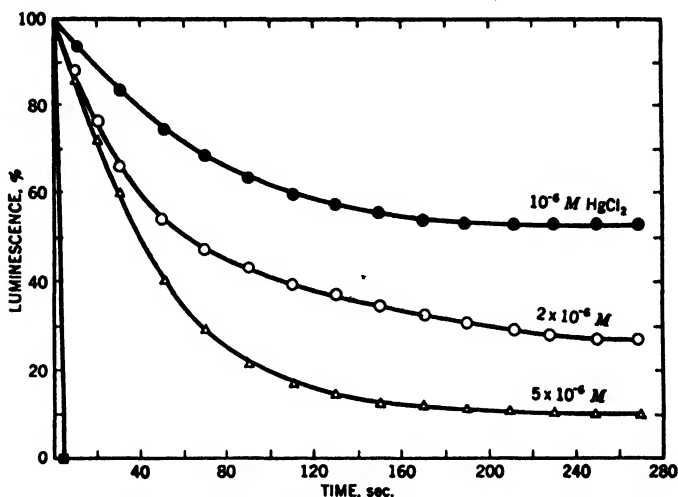


Fig. 6. Course of luminescence after adding mercuric chloride to suspensions of bacterial cells (35).

seconds after the first contact to lower the luminescence of these bacteria to its final levels—from 55 to 10% for mercuric chloride concentrations ranging from  $10^{-6}$  to  $5 \times 10^{-6} M$  (Fig. 6). It seems probable that permeability to this salt (which lowers permeability) is a real factor, the reaction within the cell occurring rapidly as the salt permeates at a relatively slow rate.

### D. PERMEABILITY TO NITROCRESOLS

These and related substances affect oxidations and fermentations in yeasts, sea urchin eggs, and many other cells and tissues. In general, at considerable dilution the nitrophenols increase oxygen consumption and the fermentative production of carbon dioxide or of an equimolecular

amount of ethyl alcohol. At higher concentrations inhibition sets in. For our purposes a possible effect of permeability on the  $Q_{O_2}$  of eggs of the sea urchin, *Arbacia punctulata*, is of interest. Clowes and Krahle (9) in studying the aerobic oxygen consumption of sea urchin eggs report that dinitrophenol stimulated eggs so that they showed an acceleration of oxygen consumption during 30 to 60 minutes. This is shown in Figure 7. This accelera-

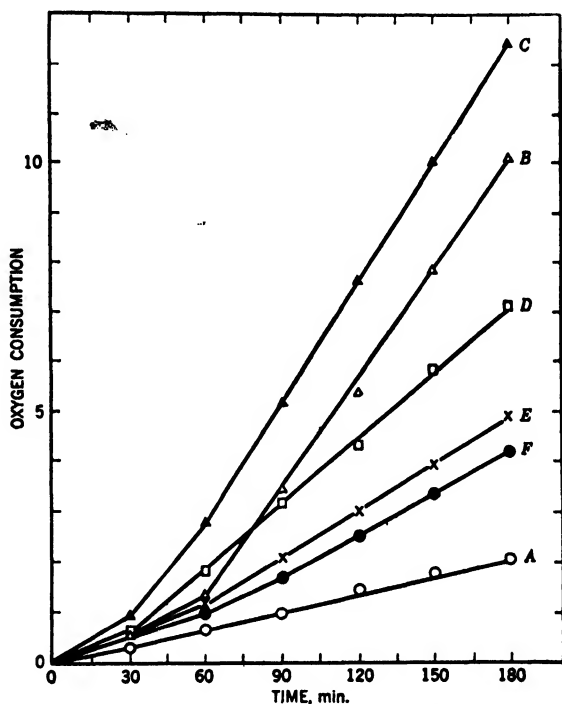


Fig. 7. Oxygen consumption by unfertilized eggs of *Arbacia punctulata* after addition of 4,6-dinitro-*o*-cresol in the following molar concentrations (9): A, control; B,  $4 \times 10^{-6}$ ; C,  $8 \times 10^{-6}$ ; D,  $3.2 \times 10^{-6}$ ; E,  $1.28 \times 10^{-6}$ ; F,  $5.12 \times 10^{-6}$ .

tion is particularly prominent when the concentration gradient across the plasma membranes of the eggs is lowest ( $4-8 \times 10^{-6} M$ ), possibly because of approaching saturation of enzymes. This result can be interpreted as showing that the full concentration of the stimulant had been essentially reached in 60 minutes. For such an egg cell, about  $72 \mu$  in diameter, assuming that the permeant diffuses quickly throughout the volume, this

figure can be estimated to correspond with a permeability of  $10^{-6}$  cm.<sup>2</sup> sec.<sup>-1</sup> This seems high in comparison with the figures established by Stewart and Jacobs (82) for the permeability of *Arbacia* eggs to, for instance, propylene glycol, *viz.*,  $0.0128 \times 10^{-6}$ , or the permeabilities to glucose referred to on pages 14 and 19. But we are led to conclude that these stimulants do penetrate at least as rapidly as this, especially since Figure 7 shows that permeation must be essentially complete at the indicated time. The nature of the effect is not known except that it appears to be related to the reactivity of the hydroxyl group of these molecules. It is not clear whether there is an actual combination of the stimulant with the enzyme, but in any event it is clear that the permeation of the stimulant is reflected by enzyme reactions. The many steps in the oxidative metabolism preclude quantitative formulations of the relation between permeation and metabolism.

The studies of Gerard (24) on oxygen consumption of *Arbacia* eggs, which may appear to be of interest at this point, have been considered on page 8 and are thought to be not usable in the present study.

#### E. PERMEATION OF IONS

Farmer and Jones (19) have shown that at least one alkali metal cation, potassium, accelerates fermentation when acting on living yeast cells and on cell-free yeast extracts. Since, presumably,  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$  (in ascending order of effectiveness) promote fermentation by living yeast (42), roughly the same order of effectiveness may be assumed to occur in cell-free extracts. Willstätter and Rohdewald (93) followed by Pulver and Verzáar (71) claimed that glucose was taken up rapidly and synthesized into glycogen. Potassium was thought to be essential for this synthesis, and was taken into the cell during the first ten to fifteen minutes, and then returned to the medium up to the original level (Fig. 8). Only after glycogen had been synthesized and later broken down was glucose broken down. This was supported by the fact that carbon dioxide production became apparent only after six to eight minutes, while over 90% of the glucose was absorbed in 15 minutes as in Pulver and Verzáar's experiments. Leibowitz and Kupermintz (43) extended the work to fermentation by *Escherichia coli* and emphasized the idea that potassium is necessary for glycogen synthesis and is liberated during breakdown of sugars. Throughout there is lack of direct proof of the presence of glycogen as shown by the negative direct tests of Mirski and Wertheimer (57) and the same authors note the early formation of esters with phosphate, that is, within one or



two minutes. This suggests that phosphorylation is the first step followed by the progress of reactions, one leading to a polyose, possibly glycogen, which would liberate phosphate, and to another reaction, the phosphorylative breakdown of glucose.

The effect of alkali metal ions, notably potassium, seems to be on the latter process. The shape of the Pulver-Verzár curves (71) suggests this possibility. Farmer and Jones (19) note that the maximum stimulation by potassium occurs when fermentation "has proceeded only to a slight extent."

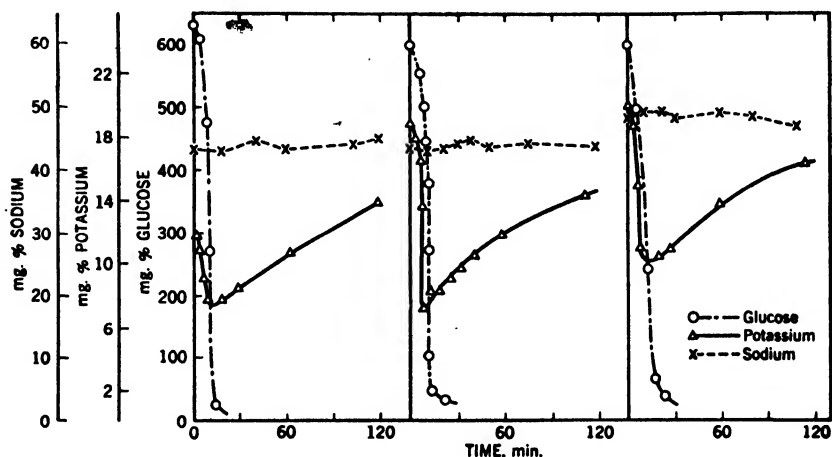


Fig. 8. Concentration of glucose, potassium, and sodium in the medium in which fermentation by yeast was in progress (70). Three successive runs with the same yeast and medium; each group of curves was initiated by addition of glucose.

Rothstein and Haege (74), studying the first ten minutes of fermentation by bakers' yeast, point out that, if enough glucose is present, the potassium enters the cells and does not leave them, while, when there is less glucose in the medium, this reverse movement of potassium occurs. Starved cells show only the reverse movement of potassium. The observation by Rothstein and Haege that one-fourth (or less under anaerobic conditions) of the glucose was stored as reserve while the remainder was fermented, supports the concept of simultaneous fermentation and synthesis. Moreover, the same authors find that the movement of potassium is primarily by ion exchange for hydrogen ions, the acid being apparently one of the intermediate products of fermentation. Rothstein (73) suspects, on the

basis of preliminary tests, that the acid in question is succinic acid. Conway and O'Malley support the ionic exchange idea (13) and suggest, without making direct tests, that pyruvic acid is the acid responsible for the hydrogen ions. Hevesy and Nielsen (30) have measured the distribution of radioactive potassium, both inward or outward, and found considerable transfer, but the rates seem low compared with those found by Rothstein and Haegge (74). Hevesy and Nielsen made no observations in the first ten minutes of fermentation, and it is difficult to utilize their results in this connection.

It appears that potassium and other alkali metal salts act on some intermediates or enzymes by momentary or more lasting combination with them. Phosphorylation seems to occur as the first step after the permeation (57). A hexose potassium phosphate might conceivably occur and be important. In this event this ionic combination, traditionally rapid, might follow the course of phosphorylation, which in turn could be the second of two successive unimolecular processes.

However, the relation between the potassium concentration and that of the hexose potassium phosphate must depend on the dissociation of the second hydrogen, whose constant appears to be normally  $2 \times 10^{-7}$ . If this were true and if such a compound occurs, then the amount of potassium taken in should be about one-half the number of glucose molecules phosphorylated at the pH of the cytoplasm, if its pH were about 6.8, that of many types of cells tested. It is probably more acidic than this, and less potassium would be used as a partner of this acid. Rothstein and Haegge calculated that one-third the glucose not stored was equimolecular with the potassium taken in. Obviously unknown factors at least complicate this hypothesis and forbid its quantitative application.

It seems more probable that the intake of potassium, as shown in Figure 8 from Pulver and Verzá, reflects the counter gradient of hydrogen ions. As fermentation proceeds and before carbon dioxide is liberated, the hydrogen-ion concentration in the protoplasm rises, and with this the rate of ionic exchange rises. Although potassium is essential for maximum fermentation, there is no evidence that its permeation is normally slow enough to affect the fermentation rate curves. In fact such experiments as those done by Brooks (5) demonstrate exceedingly rapid penetration of potassium and other cations by ionic exchange into several types of cells. Rothstein (73) shows that, when no potassium chloride or similar salts are present in the medium, glucose can be taken up (see also Conway and O'Malley, 13) with diffusion of an acid without ionic exchange. The loss of

an intermediate would affect the yield of fermentation, and would be prominent when potassium was lacking. The loss was especially conspicuous when the anion in the medium was citrate, which appears to increase cellular permeability. Here permeability does affect enzyme reactions, but no appropriate figures are available for a closer study.

#### IV. Summary

The theoretical bases for the study of the relation between permeability and enzyme reactions were first studied in some detail. It seems that enzyme reactions can be followed in accordance with the laws of mass action, provided that such variables as those introduced by pH, inhibition, and autocatalysts are known and taken into account. Aside from this not always simple requirement, most of the difficulty in this inquiry lies in the complex metabolic chains and networks. It is often true that it is impossible to trace with confidence the relation between permeation and the measured quantity, a relation mediated through many steps. Burton, in two papers, has presented a good analysis of reaction chains, and has offered equations which may prove helpful when it becomes possible to delineate the metabolic steps.

Weichherz was apparently the first to utilize the concept that permeation can be treated as a reversible unimolecular reaction. However his equations assume a direct one-step relation between permeation into yeast cells by glucose and the liberation of carbon dioxide. But it seems that several steps intervene, four or probably several more. The sigmoid curves observed by Nord and others for this end result seem to result from phosphorylated processes occurring when glucose penetrates the yeast cell.

Gerard's calculations relative to the oxygen supply and respiration of cells like eggs of sea urchins, while empirically valid, have a weakness, frequent in other published work (Krogh, Hill), in neglecting the existence and effectiveness of the plasma membrane. Therefore these calculations are not useful in this field.

The geometrical shape of cells has been shown not to be important. If the plasma membranes, which are thin, control permeation, the process of permeation would be exponential. Since permeation of all, or all except the smallest, cells studied can be treated as exponential curves, all such curves can be treated mathematically, like unimolecular reactions. The structure and probable thickness of plasma membranes are described as consisting of micelles of lipid materials enmeshed in a layer of extended protein molecules which attach more or less to each other.

It is difficult to find the experimental data for testing the concepts discussed in this paper. Most of these data are found in figures and tables in papers devoted to other aspects, and permeability is usually not mentioned or indexed. It is hoped that the few instances discussed will represent the field, but there is no expectation that this array is complete—it is virtually impossible to make this complete.

The penetration of glucose seemed to offer the opportunity to trace the effects of permeability. But for reasons mentioned above it has proved impossible to trace any such relation. In fact, recent work shows that glucose penetrates yeast cells almost completely before carbon dioxide appears, so that the induction period, which is longer than the period of glucose penetration, cannot be a direct result of that permeation.

If calculations are made which correlate carbon dioxide production in the relatively steady state of fermentation with rate of permeation by glucose, it can be concluded tentatively that the rate of fermentation is regulated by a permeability of plausible magnitude. This seems to be a reasonable conclusion, *i.e.*, that, as fast as glucose arrives by diffusion through the plasma membrane, it flows through several steps toward the final product.

The effect of a narcotic, notably ethylene, can be referred to its reaction with (adsorption on) one or more enzymes, or with equal justification to its effects on permeability. Since there is conflicting evidence on the effects of narcotics on permeability, no effect can be considered independent of permeability changes. The narcotics, however, are related in at least two ways to such functions as the oxygen consumption by many types of cells or the bioluminescence of bacteria. Considering evidence on this point, it seems quite possible that permeability controls one of these relations.

There is good evidence that mercuric chloride reacts with enzymes as well as with the plasma membrane, and the relatively slow onset of its effects on bioluminescence reflects one or both of these roles. Similarly, the onset of stimulation of oxygen consumption by nitrophenols shows that permeability at first limits the effect, an effect disappearing in about a half hour.

The effects of certain ions, ordinarily potassium, on the fermentation process of yeast are of a complex nature. Apparently the beginning fermentation produces acids whose hydrogen ions enter into ionic exchange with the potassium ions of the medium, and the potassium thus obtained facilitates in turn further fermentation. Quantitative figures, as well as knowledge of the steps involved inside living cells, limit us to only pro-

visional conclusions concerning relations between permeation of reactants and enzyme reactions within living cells.

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# THE PROPERTIES OF PROTOPLASM

## With Special Reference to the Influence of Enzymic Reactions

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### I. Introduction

The chemist is often baffled by protoplasmic reactions, for at times the living substance appears to employ means quite unknown to him. For example, electric potentials are developed in organisms without the use of metals which are necessary in the laboratory for the generation of electric potentials of equal magnitude. Certain other highly complex reactions occur in organisms which are fully understood. Osmosis is one of these; its laboratory counterpart is probably a very close duplicate of the natural process. Even so intricate a series of reactions as that involved in respiration has been elucidated with fair success, chiefly because of our increasing knowledge of enzymic reactions. Many vital processes, however, still



remain obscure. These complex and as yet unfathomable protoplasmic reactions probably all involve enzyme activity. In fact, the catalytic action of enzymes is as near an approach to life as exists in the nonliving world, and for this reason the biologist often turns to catalysis as an explanation of vital processes. Perhaps he does so too frequently, but he is sometimes left with no other choice, for catalysis is often not only the most likely but the only conceivable explanation of a vital reaction. I have long wondered by what means protoplasm accomplishes its extraordinarily rapid and pronounced changes in viscosity. In the case of gelatin, an extreme change in viscosity is brought about by a change in concentration or in temperature, but the living cell attains the same result without change in either concentration or temperature. A close approach to the vital method of accomplishing viscosity changes is to be found in the coagulating effects of certain enzymes such as rennin. Salts may play their part as they do in the thixotropic behavior of gels, but salt solutions are static systems. Thixotropic changes in ferric oxide or bentonite are not autoreversible, whereas viscosity changes in protoplasm are. The catalytic action of enzymes makes such processes in living matter possible.

Each fact and each hypothesis in this review of the properties of protoplasm is presented quite independently of enzymes, but when experimental evidence indicates that catalytic action is probably involved, then this is stated.

## II. Physical Properties and Phenomena

### A. VISCOSITY

The viscosity of nonliving systems (13,38) and protoplasm (81) has been so thoroughly and so often reviewed that it will be sufficient here to refer briefly to some recent work.

The situation in regard to the viscosity of body fluids remains ever the same; when the fluid is serum the viscosity values are accurate, but when blood is studied the values have little meaning because blood out of the body is no longer normal blood. Although there is still much discussion on the significance of the values, clinical measurements continue to be made (29). The measurement of the viscosity of protoplasm presents difficulties similar to those of blood viscosity measurements. The methods are numerous (81), and, though they are not of the degree of accuracy required by the physicist, the values are nonetheless fairly good. It is rather the role of protoplasmic viscosity in physiology and the meaning of the wide range in values which present problems.

Many competent investigators regard viscosity as one of the most significant of protoplasmic properties. On it, they say, do such basic activities as amoeboid movement, cyclosis, and metabolism depend; but, this is only indirectly true. It is contractility and not viscosity on which protoplasmic movement primarily depends; the two properties are independent variables. Viscosity plays its part in protoplasmic movement, but it is not a cause. If the force responsible remains constant, the rate of movement will be inversely proportional to the viscosity of the protoplasm; but, if the motive force varies, the rate of flow will vary in direct proportion to it, and the viscosity need not change at all. The situation is similar in the case of metabolism; viscosity plays a secondary part. Lhérisson (44) has pointed out that metabolic rate is inversely proportional to viscosity. He bases this conclusion on the fact that the rate of chemical reactions is dependent upon diffusion, and rate of diffusion is proportional to viscosity.

### *1. Anomalous Viscosity*

Anomalous behavior is often more revealing than normal behavior. To be sure, enough systems must behave in accordance with laws set up by physicists and chemists so that our world shall be recognized as an orderly one. Once this requirement is met, the anomalous systems become those from which we learn. This is true of anomalous viscosity, anomalous osmosis, anomalous or "negative" adsorption, and a variety of other irregular happenings in nature. One must, however, realize that a system is anomalous in behavior or a reaction is negative only because of our ignorance of what is actually happening. Thus, if adsorption is defined as the concentration of a substance at an interface, it is always a positive and never a negative action. The quantity adsorbed may be exceedingly minute, but it cannot be less than nothing. For example, if we are measuring the adsorption of a solute when it is the solvent that is being adsorbed, we erroneously term the process "negative" adsorption.

Anomalous osmosis presents a similar situation. We expect a certain result, namely, the movement of a liquid in conformity with the laws of diffusion, and get instead movement in the reverse direction; so again we say, "anomalous" osmosis. But again it is some other force, of which we are unaware, which upsets the expected situation and causes diffusion against a concentration gradient. The "anomalous" osmosis is in full obedience to some other force, which may be an electric potential.

Once an "anomalous" situation is understood it ceases to be disturb-

ing; and we find, for instance, that the laws of viscosity still hold, although they are interfered with in some ways. Other forces or structural features prevent normal flow (by normal is meant the expected behavior of an orthodox fluid). The anomalous flow of certain colloidal systems, notably organic jellies when fluid, has been a field of extensive investigation. Bingham (13), Freundlich (19), and many others have discussed non-Newtonian viscosity in full.

Anomalous viscosity has revealed more of the structure of fluids, and of such physical properties as birefringence, elasticity, and thixotropy, than any other single physical characteristic. The physicist had stated the laws of normal flow, but it remained for the colloid chemist to investigate the non-Newtonian, "nonlawful" fluids which exhibit anomalous viscosity. The Society of Rheology was a direct result of interest in the non-Newtonian behavior of liquids. Indeed, it may well be said that the *raison d'être* of colloid chemistry is the digression of physical and chemical systems from classical laws.

Substances which exhibit anomalous viscosity have been called quasi-viscous. Other designations, in which attempts are made to characterize and in part explain anomalous viscosity, have been used. They are variable viscosity, structural viscosity (61), and plasticity. Of these expressions, plasticity is the most unfortunate for it is precisely a lack of plasticity which characterizes non-Newtonian sols and gels. It is confusing and misleading to redefine a familiar term. Certain chemists such as Guth (31) use plasticity in its older and correct connotation.

The structural causes of anomalous viscosity have been little discussed, perhaps because they are not generally recognized. My own interpretation, presented quite some years ago at the inaugural meeting of the Society of Rheology, is as follows: non-Newtonian fluids are elastic and the structural feature responsible for the one, non-Newtonian, property is responsible for the other, elastic property (79). So far as I am aware this postulate still holds. Its full meaning will be better understood when protoplasmic structure is discussed.

Experimental evidence that protoplasm belongs to the large group of organic substances which are non-Newtonian in behavior is meager, but the indirect evidence is ample. The chief constituents of protoplasm, the proteins, show anomalous viscosity. Among the numerous indirect experimental proofs of the non-Newtonian character of protoplasm is that of flow birefringence. Pfeiffer (67) has shown that streaming protoplasm, when viewed between crossed Nicol prisms, is anisotropic, that its flow is

anomalous and therefore not obedient to the laws of Newton, Poiseuille, and Stokes, and that the structural features responsible for the refractive behavior of protoplasm are also responsible for its anomalous viscosity.

Northen (61,62) has done much work on the structural viscosity of protoplasm by centrifuging cells. The percentages of *Elodea* cells in which the chloroplasts are displaced, *i.e.*, moved to the centrifugal wall, are determined. The number of displaced chloroplasts in control leaves averages 12%, whereas the chloroplasts of leaves treated with 0.5% ethylene are displaced in 48% of the cells. The difference, 36%, is a negative value and therefore indicates a decrease in protoplasmic viscosity. Ethylene, chlorhydrin, thiourea, ether, ethyl alcohol, propyl alcohol, butyl alcohol, cupric chloride, and zinc sulfate decrease the structural viscosity of protoplasm when the exposures are not over two hours. Northen assumes that decrease in viscosity results from the dissociation of protoplasmic proteins, that is, from the splitting of large protein molecules into units of smaller size. He hypothesizes (62) that protein dissociation often conditions increases in the rates of cell processes, such as respiration, imbibition, permeability, polysaccharide hydrolysis, and that under some conditions protein dissociation may be responsible for the breaking of rest periods. The idea that a disaggregation of cell colloids, resulting in greater dispersion, hastens the rate of cellular processes is a well-established hypothesis. Northen's concept augments this older idea in that he recognizes that dissociation not only increases the surface but may also result in the liberation of enzymes from their previous combinations and in the formation of enzyme activators such as sulfhydryl groups.

It should be borne in mind that the concentration and the time of treatment of an agent, whether an anesthetic agent, a salt, an alcohol, or temperature, alter the final results; certain concentrations often first lower then raise the viscosity of protoplasm. And there is always the question: do all forms of protoplasm react in the same way? Sometimes this is the case, extraordinary as it may seem, and sometimes there is considerable variation, the cells falling into one of two classes. Northen (62), corroborating Heilbrunn (33), states that the protoplasm of *Spirogyra* is a type which regularly shows an increase in viscosity with diminishing temperature, whereas *Amoeba* is an example of the type which exhibits a decrease in viscosity as temperature is lowered. The effect of sugar on the structural viscosity of protoplasm is interesting and somewhat surprising. Sucrose, like potassium chloride, when plasmolyzing an *Elodea* cell induces a striking decrease in viscosity of the protoplasm.

## 2. Gelation

Gelation represents an extreme change in viscosity, but the mechanism of gelation is quite different from a simple increase in internal friction such as is responsible for an increase in viscosity. Gelation is the result of an interlocking of linear molecules.

If we use the term gelation in its broadest sense, then reversible gelatinization and irreversible coagulation are both included. Usually, when living material is under consideration, gelation implies only gelatinization. The reversible gelation of protoplasm produced by electrical stimulation of *Amoeba* was referred to by Bayliss (7) twenty-five years ago. A naturally occurring reversible gelation in *Amoeba* is thought by Mast (52) and others to be the mechanism of amoeboid locomotion. Claude Bernard regarded "coagulation" as the mechanism of anesthesia; obviously it must be reversible if there is recovery.

## 3. Thixotropy

When reversibility, both solation and gelation, is rapid—often instantaneous—it is known as thixotropy, a term introduced by Freundlich (20,22) and much used by him (21,23) and others (77,79) to explain biological processes. Thixotropic behavior is not exceptional; in fact it is very common provided suitable concentrations of colloid are chosen. Examples are aqueous thixotropic gels of the oxides of aluminum, iron, scandium, vanadium, titanium, thorium, etc. (93), colloidal bentonite (25,32), myosin (18), dibenzoylcystine (98), and barium malonate in a medium of water and alcohol (98). Aqueous solutions of gelatin exhibit thixotropy (24), both as concentrated sols and as gels (23). The instantaneous collapse of nonliving thixotropic gels is brought on by mechanical agitation, but in living protoplasm thixotropic disintegration takes place spontaneously, or it may be brought on by shock (88) and then it has every resemblance of collapse due to "nervous" shock.

## B. ELASTICITY AND RELATED PROPERTIES

**Elasticity.** Protoplasm is elastic, usually highly so, but occasionally young protoplasm is apparently devoid of elastic qualities; at such a time the living substance is plastic. Treitel (95) has called attention to the change from plasticity to elasticity which takes place with age in plant tissues.

The bearing of elasticity on structure has been frequently referred to by Scarth (70) and myself (81). In Scarth's words, "elasticity is the chief quality of protoplasm on which a conjecture of the ultramicroscopic structure of living matter can be based." But our interest in elasticity need not

begin and end with its bearing on the structure of living matter. Elasticity is in itself worth measuring. Norris (59,60) determined Young's modulus for a strand of myxomycete plasmodium, and found it to be  $9.0 \times 14^4$  dynes cm.<sup>-2</sup> at room temperature, for increases in length up to 40%. With increase in temperature the value of the modulus is lowered. With decrease in temperature, to 10°C., there is an increase in the value of the modulus to over twice that found at room temperature, and the protoplasmic strands no longer follow Hooke's law.

An important observation by Pease (66) answers a long controversy on the place where the elasticity of a cell is situated; it is not concentrated at the surface as has been frequently maintained. This observation by Pease (66) is in agreement with a contention I have long held (81), namely, that the elastic properties of protoplasm are distributed throughout the substance and do not reside merely at the surface. This fact in no way detracts from the reality and significance of surface elasticity (43).

Pease (66) also found that chloretone anesthesia has no effect on the elasticity of strands of the slime mold *Physarum*, although streaming is reversibly stopped. Pease should explain whether he means the modulus of elasticity or a coefficient of extensibility. If he means the former, it is surprising that anesthetized protoplasm has the same modulus of elasticity as normal protoplasm, for with anesthesia comes gelatinization. In all my experience (85) the cessation of streaming when brought on by any one of a number of anesthetic agents is accompanied by thixotropic setting, the rapid reversible gelation of the protoplasm. If this is true for chloretone it would mean that the modulus of elasticity is the same for the protoplasmic gel as it is for the protoplasmic sol, a very unlikely conclusion.

**Extensibility.** Some years ago Freundlich and the writer (26) studied the elasticity of gelatin; the method used involved the attraction of a minute nickel particle by an electromagnet. The method was then applied to echinoderm eggs (78). In this work Freundlich and the writer determined elasticity in terms of extensibility. The two properties are not synonymous. In general, substances which have a high modulus of elasticity have low extensibility or stretch (*e.g.*, steel), and substances which have a low modulus of elasticity have high extensibility (*e.g.*, rubber). The limit of extensibility is a significant value for elastic jellies but it is not a measure of what physicists call the elastic limit. Treitel (95) is now at work on an analysis of the elasticity, extensibility, contractility, flexibility, plasticity, and related properties of organic material, both living and non-living. He makes clear the distinction between steel and rubber elasticity.

All students of this general problem should become familiar with the work done on the structural features of rubber, notably by Mooney (57), Treloar (96), Guth (31), Meyer (53), and Mark (48).

Plowe and the writer (89) determined the effects of salts on protoplasm and found that calcium increases extensibility while sodium diminishes it, and magnesium has no effect at all. The following lyotropic ionic series was obtained:  $\text{Ca} > \text{Sr} > \text{Mg} > \text{K} > \text{Li} > \text{Na}$ .

**Contractility.** There is no fundamental difference between elasticity and contractility when inanimate material such as rubber is under consideration. A stress-strain curve will, on compression, extend below the line of zero stress and will extend above on stretching. But if we are dealing with living matter of the type of muscle, this may or may not be true. The negative temperature coefficient of rubber is not equivalent to the heat contraction of muscle. The Gough-Joule effect (37) need not, therefore, hold for muscle. If heat is applied to muscle, it will contract, but cold does not cause living muscle to expand. This is true because we are dealing with living matter, a dynamic mechanism with self-regulatory powers, and not a passive piece of material. In muscle, any change in metabolism sets up a new state as a result of which tension is developed. The tension thus produced is the result of a new situation, literally a new system, an active state, which was formerly not present.

Although the foregoing distinctions between a living dynamic machine such as muscle, in which, on contraction, a new system and not simply a new equilibrium arises, and a nonliving organic gel such as rubber, with its high extensibility, there is a pronounced similarity in the mechanisms involved. In other words, muscle, though it is a living machine, possesses elastic qualities—because of its main constituent, myosin—which are fully comparable to those of rubber; therefore, the Gough-Joule (37) effect may hold. But in living muscle the effect is completely masked by other qualities of muscle functioning as an independent heat machine. This deduction holds for all irritable tissues. In connective tissue, such as tendon, the laws of rubber elasticity will apply far more accurately.

The foregoing state of affairs appears to hold for living muscle. As myosin muscle is and must be extensible, but, as a living machine, it is contractile only. We are forced, therefore, to question the work of Hill (35) and others in which it was shown that muscle in the relaxed state resembles rubber, that the stretch-strain curves of the two are similar, that the stretching forces are of the same order of magnitude, and that both rubber and muscle exhibit the same anomalous thermoelastic behavior (at

small extensions muscle absorbs heat and at increasing extensions an inversion point is passed above which muscle, like rubber, generates heat). That the foregoing results have been obtained by able physiologists supports my contention that muscle as myosin is rubber-like. For some inexplicable reason in the experiments of Hill those qualities of muscle which reside in the protein myosin dominated those other qualities which muscle possesses by virtue of being living tissue. Living muscle functions as an autonomous heat machine and as such the thermoelastic behavior claimed for it by Hill cannot hold. And so we must conclude that the contraction of activated muscle is not the same thing as the contraction of rubber nor is the difference due solely to the complexity of muscle as compared with the relative simplicity of hydrocarbon rubber, but to the fact that activated muscle is not just muscle in a new equilibrium but is a new system.

Contractility not only plays a prominent role in muscle but it is a basic property of all protoplasm. Because muscle is made of protoplasm, which is highly contractile, a muscle fiber may contract to one-tenth of its original length, a contraction of 90% and a deformability equal to that of the best rubber.

Contractility takes a special form in some organs and organisms where it is rhythmic. Rhythmic contractility (82) is probably a potential property of all forms of protoplasm, manifesting itself primarily in tissues where it is needed, in heart muscle, intestines, the diaphragm, and in many unicellular organisms such as leucocytes, swimming protozoa, and myxomycetes (40).

The molecular basis of contractility is now quite well understood. It is generally assumed that elastic proteins and rubber-like materials are built of flexible polypeptide chains. The similarity in the molecular mechanism of such materials is indicated by the fact that all yield S-shaped curves. Such curves are obtained for gelatin and glue, and by Treitel (95) for cellulose in the living plant.

How the contractile molecules operate is shown by Astbury (2,3,4) in his work on wool. Under exposure to steam, wool fibers become shorter by supercontraction, which means that the normal, partly coiled state of  $\alpha$ -keratin folds up still further. A slightly stretched state is apparently the normal state of equilibrium for rubber; this permits both stretching and shortening of the molecular threads.

Again let me remind the reader to become familiar with the excellent work now being done on the elasticity of wool (2), rubber (31,48,96), and like materials.



## C. SPIRALITY

Spiral form, growth, and movement are very common in nature, so much so that I have regarded spirality as a property common to all living matter (80). The movement of protozoa and men (73), the growth of trees (80), molluscs, and body parts such as horns and tusks, and the shape of chromosomes (41), are spiral. To what extent spirality in animate nature is to be attributed to the asymmetry of crystals, the asymmetry of the carbon atom, or helical organic molecules is a matter of conjecture, but the spirality itself cannot be doubted.

**Torsion.** Studies of spiral growth and movement led me to the belief that a state of <sup>or</sup> torsion is established in protoplasm when it is in motion, provided certain simple conditions are met. These conditions exist in a freely suspended strand of protoplasm in which flow is only in one direction at one time. Experimental verification of the assumption that a state of torsion exists in protoplasm when in motion was accomplished by attaching a delicate glass needle to the lower end of a freely hanging strand of protoplasm. The needle served as a pointer to indicate the direction and extent of torsion in the thread. There would of course be no reason for the swing of the needle, first in one direction and then in another, were there not an alternating twist in the protoplasmic thread, and there would be no reason for the twist were not some force bringing it about. This force is the movement of the protoplasm, and it will bring about a twisting of the strand only if it takes a spiral course or moves in a tube with a spirally wound wall. I assumed the latter to be true, for there was no visual evidence that the protoplasm takes a spiral course, and there is much evidence of spiral structure in cell walls, both specific and general, as indicated by the widespread occurrence of spiral structure in nature (36,81).

An apparent lack of synchronism between the swing of the needle and the flow of the protoplasm proved an interesting and disturbing problem. The period of swing of the needle was but half the period of flow, that is, at each reversal in direction of flow there was a corresponding reversal in direction of swing of the needle, as there should be, but there was also a reversal in direction of swing of the needle while flow continued in one direction. This was interpreted as follows: there is a gradual increase in torsion as a result of an increase in rate of flow up to the midpoint of the shuttle movement in one direction; after this, there is a gradual lessening in rate of flow and therefore in force applied, and a consequent unwrapping of the protoplasmic thread while flow continues still in the same direction, but at an ever decreasing rate.

#### D. PERMEABILITY

To review the subject of protoplasm without mention of permeability would be nothing short of scientific sacrilege, but permeability has been so often (81) and so well reviewed (14) that a brief mention of some recent contributions will be sufficient here.

An erroneous statement once printed must thereafter be repeatedly denied. Such is the mistaken idea that only molecules, and not ions, enter cells, an almost inconceivable view in the light of modern ideas on solutions. The statement was based on experiments made with carbon dioxide, and certain other substances such as cyanide with which tissues normally have nothing to do. Lundegårdh (47) denies that materials enter cells only as molecules. The reviewer has long felt that in the face of the modern concept of the complete ionization of salts, even at high concentrations such as are never reached in the soil, it is impossible that most elements should enter cells as molecules, for of many essential salts in the soil there are no molecules present. To call attention to the fact that carbon dioxide is a weak electrolyte and therefore but partially dissociated is of no avail, for carbon dioxide is but one of many substances which enter cells. Lundegårdh states: "If the cell or organ is placed in contact with a solution of a single salt, full equivalency in the absorption of cations and anions is an exception.... These experiments show that the salt is not taken up as molecules..." Krogh (42) adds further evidence in support of the exchange of ions.

The old controversy of protein *vs.* lipide at the cell surface is given a point in favor of protein by Valko (97). He says: "... biochemical effects of surface-active agents are to be partially explained on the basis of their affinity for proteins. This causes combination, resulting in upsetting the balance of electrostatic forces and non-Coulombic cohesion in the molecule and changing the interaction of proteins with solvent molecules. As a further consequence, the bonds between components of conjugated proteins may be disrupted. Denaturation and unfolding of protein molecules and inactivation of enzymes, viruses, and bacteria result."

#### E. ELECTRICAL FORCES

One of the great problems in biology is the cause and function of electrical forces in living matter. It is hardly possible to deny their existence. The electric eel and ray fish both give ample evidence of the presence of electric potentials in organisms. Viewed theoretically, if electrolytes are separated by a selectively permeable membrane, a difference in potential

between the two sides of the membrane must inevitably result. Such an unequal distribution of ions may be interpreted as a Gibbs-Donnan equilibrium. There can, therefore, be no question of the presence of electrical forces in tissues but there is a question as to their function. Of this, little can be said. One of the processes in which electric potential is most likely involved is nerve conduction. It is true that we measure a difference in energy level (in electron pressure), and because we measure a difference in chemical potentials with electrical instruments we speak of electrical energy. We may as well have viewed it as chemical, thermal, or mechanical energy. The paths represent mechanisms, and we are at liberty to postulate any mechanism for purposes of thermodynamic formulation, provided that it does not violate the fundamental postulates of thermodynamic theory. Our postulates give no clue as to which of the mechanisms is the actual one. Because we pick up some of the free energy by means of platinum electrodes and thus produce an electric current that can be measured, it is not implied that an electric current is produced in the system. In other words, we measure and express difference in level of free energy in terms of an electric potential simply because the potentiometric method is a convenient one for measuring difference in energy level. We could just as well express the free energy in terms of a different scale—say, for instance, in terms of calories.

Electrical forces in tissues are most often attributed to the unequal distribution of ions at membrane surfaces, but equally if not more probable as a source of electrical energy is the untold number of oxidation-reduction reactions which take place within a cell. These reactions account for the extraordinary capacity of living systems to generate potentials of rather high magnitude without the metals required in a galvanic cell.

There is also the question of electron flow in tissues. This has been said to occur along nerve fibers (45,46), but our knowledge of nerve conduction as a mechanism is very incomplete. Actually, there does not appear to exist in living systems any mechanism that is analogous to the metallic electron conductor.

Of the several forms of electric potentials in living matter—concentration, diffusion, liquid junction, oxidation-reduction, membrane, and injury potentials—that due to oxidation-reduction equilibria is the most likely energy source and one in which catalysis, possibly autocatalysis is involved.

The amount of research done on electrical forces in organisms, especially in nerve conduction, is rather great, but many questions remain, one

of which is the chemical nature of the substance primarily responsible for tissue potentials. All agree that the potential arises at surfaces. Cell membranes, when substantial, are usually regarded as consisting of protein material for they show amphoteric qualities. It is equally true that these morphological membranes are coated with lipides which materially affect electrical forces. Using models, Beutner (12) concludes that lipides are the cause of bioelectricity. As far as the chemical basis of electric potentials, when one can have a protein, does seem a bit extraordinary.

Toxicity is a little understood subject, but there are numerous suggestions in explanation of it; among them is surface tension with such contradictions as the fact that chloroform, the stronger narcotic, has less effect on surface tension than ether, a weaker narcotic. Beutner (12) regards electrical changes in living tissue as the cause of drug action. He says that, when an animal collapses after a fatal dose of strychnine, atropine, or other alkaloid, the cause of its death is an electrical injury to its brain, as if the organism had received directly an electrical shock, had been struck by lightning, or had been electrocuted.

Among the many research problems in electrokinetics, the latest is that on brain waves. Beutner (11,12), Barnes (5,6), and Burr (16,17) have been especially active in this field. Brain waves were not discovered in the human brain, as generally supposed, but in the brain of the rabbit. The waves are conducted out of the skull and through the skin by means of wires which touch the scalp. Only eight-tenths of the voltage is lost after leaving the brain cells. Normal brains pulsate electrically at a frequency of ten to twenty a second. Ten per cent is the rate of most healthy persons. The rate is slower in babies. The brain waves of an unborn child have been recorded through the mother's abdominal wall and found to be four per second. The potential varies from 5 to 75  $\mu$ v. (6).

In general, fast, low-voltage waves are found in nervous people. Very slow waves, less than eight per second, are a bad sign, and occur in 50% of criminals and in 70% of delinquent children. Sleep slows the waves, but the mind never rests, for in deep sleep the electrical pulsations continue unceasingly. Fatigue, fear, disease (epilepsy), opiates, and stimulants alter brain potentials. Brain wave frequency has been correlated with anesthetic agents. So close is the correlation between brain waves and body activities that the determination of the potentials has become common in medical practice, and manuals on clinical electroencephalography have been published.

The psychology of brain waves is a tempting field, but with much contradiction. Any form of psychological correlation is opposed by the psychoanalysts. They question all correlations between brain wave frequency and mental effort. Fast frequencies are said to be common in schizophrenics.

Burr has correlated potential differences with development (16) and growth (17) in both plants and animals. Barnes (5,6) has applied tissue potentials to the problem of healing rate, and finds that loss of positive wound potentials affords a delicate indicator of the generation of traumatized tissue.

#### F. RADIATION

The subject of radiation may be considered as a problem of radiation from protoplasm or of the effect of radiation on protoplasm. The latter offers little, for in spite of a great amount of research the results are vague, varied, and contradictory. For example, work with ultraviolet light is of little value unless the wave length is known and restricted to a very narrow band; certain bands in the ultraviolet are beneficial, certain others harmful, and some lethal; therefore, when the entire ultraviolet spectrum is used, an average of all effects is obtained. In general, ultraviolet light of wave lengths 2550 to 1800 Å is damaging to protoplasm, and lethal in time.

The effect of x-rays on protoplasm has likewise yielded little that is definite (author's unpublished data). More successful, but still not well systematized, is the extensive medical x-ray treatment of tissues. The more closely this medical work approaches biological methods the more precise does it become (94).

Radium appears to have a more specific influence on protoplasm. As slime mold protoplasm approaches radium, it first hesitates at a definite distance (about 5 mm. from a 12-mg. needle of radium), then slowly advances, retreats, advances again, and stops; it then goes all the way to and over the needle, and finally retreats to a safe distance which is maintained from the radium on all sides.

Tansley, Spear, and Glückmann (94) have shown that cellular degeneration is produced by small doses of gamma rays and that this degeneration is due to an effect upon dividing cells which unfits them for mitosis. Spear and Tansley (91) find similar results from the action of neutrons on the developing rat retina. Radioactive elements, particularly the artificially produced isotopes, have of late been intensively studied in regard to their penetration, translocation, and effects on protoplasm. Brooks (15) and Mullins (58) find that some of these isotopes (Na, K, Rb, PO<sub>4</sub>) are not injurious when the radioactivity of the solutions used lies below poorly defined levels. Experiments using elements of sufficiently low activity yield no different results from those with nonradioactive salts, especially in re-

gard to permeation. In contrast is the medical work which has shown that radioactive strontium and phosphorus are selectively absorbed in bone.

The use of tracer substances, or so-called "tagged" atoms, promises much. Spear (90) describes radioactive sodium, an emitter of gamma rays, given to a patient by the mouth as common salt; it can be detected in the finger tips by means of a Geiger counter within two minutes after administration.

The medical man is primarily interested in the therapeutic administration of radioactive substances. Development of the technic is uneven, the furthest being in treatment with radioactive phosphorus prepared by the bombardment of red phosphorus with high speed deuterons. It is too early to judge, but artificial radioactivity is at least as good as x-ray treatment of certain blood diseases, though neither is a cure (90).

The radiation of energy from protoplasm is a very delicate subject at present. Some years ago Gurwitsch (30) claimed to have discovered that living matter radiates some sort of energy which stimulates other living matter to more active growth. A hundred or more articles appeared corroborating this work. Although nearly every European country gave support to the so-called mitogenetic or Gurwitsch rays, Americans, with two or three exceptions, doubted the results from the outset. A number of experiments were done in America with the express purpose of disproving the hypothesis, so great was the emotional opposition. Just as unfortunate were the careless experiments done in support of the hypothesis. Only one well-planned research project was carried out in a serious effort to ascertain the truth of the matter, and here the results were negative.

Two conclusions seem inescapable: first, that the experiments of Gurwitsch did not prove his hypothesis; second, that living matter does radiate energy. It is no new experience in science that an excellent idea is advanced on the basis of faulty research only to have the idea later fully substantiated by unimpeachable experiments. This was true of de Vries' theory of mutations. The only sensible way to view Gurwitsch's hypothesis is to consider it as a suggestion and ignore his experiments. Gurwitsch maintained simply that living tissues radiate a form of energy, probably ultraviolet light. Why such an idea should call forth bitter controversy is difficult to understand, when the radiation of heat from warm-blooded animals, of light from the firefly—and here the precise oxidation-reduction reaction is known—and of electricity from the ray fish are familiar phenomena. Opponents of Gurwitsch say that there is no ultraviolet

in the light of the firefly. This may be true, but surely there is no fundamental reason why the light of a firefly should necessarily stop short of the ultraviolet.

Chemists are aware of many types of reactions which radiate energy—exothermic ones, luminescent ones; and physicists are aware of many forms of radiant matter. Protoplasm is not less active than these, not less dynamic than the radioactive elements, potassium, rubidium, and even radium, which it contains. Glasser (27) has conservatively reviewed the hypothesis of Gurwitsch.

#### G. RHYTHM

Most natural phenomena are rhythmic, and, in those which appear not to be rhythmic, the rhythm may be hidden or disrupted by other forces. The movements of the planets, the seasons of the year, the life cycles of plants and animals, the beat of the heart, the streaming of protoplasm, and the pulsations of a slime mold are all rhythmic. Opposition to the idea of rhythm in natural phenomena should be replaced by skepticism when absence of rhythm is claimed. If natural phenomena which constitute the environment of organisms recur rhythmically, as do heat and cold, rain and drought, short days and long days, cosmic radiation, etc., protoplasm would, in such an environment, inevitably develop rhythms in growth, reproduction, and metabolism, and these would lead to other rhythms as, for example, in movement. Among rhythmic activities is the shuttle flow of the protoplasm of myxomycetes; its cause, the pulsation of the plasmodium, is likewise rhythmic.

The protoplasm of a slime mold (*Physarum*) flows first in one direction, and then in the other. If such a plasmodium is photographed every five seconds on a moving picture film and shown at the usual rate of sixteen frames per second, optically speeding up the apparent rate of movement of the slime mold eighty times, the plasmodium is seen to go through rhythmic contractions and expansions which are synchronized with the outward and inward flow of the protoplasm. Protoplasmic movement in slime molds appears, then, to be due to the rhythmic contraction and relaxation of the plasmodium, with a total, in *Physarum*, of 95 seconds for each complete pulsation, 45 seconds for systole and 50 seconds for diastole; the additional 5 seconds in time of outward flow account for advancement.

Three distinct rhythms in slime molds were revealed in the first moving picture taken (82). Kamiya (40) verified these, and went much further in his analysis of curves of protoplasmic flow by viewing the protoplasm of a

slime mold as a polyrhythmic system. Many rhythmic cycles are simultaneously operative in one and the same plasmodium.

Kamiya's verification of the discovery of more than one rhythm in protoplasm and his additional finding that a plasmodium is a polyrhythmic system were based on irregularities in graphs of the motive force. The force expended in producing flow was ascertained by opposing the protoplasmic movement with air pressure in a micropressure chamber of special construction (39,40). The pressure necessary to stop flow, to hold the protoplasm at a standstill, is a measure of the pressure produced by the contracting plasmodium. When this pressure, which varies with the shuttle movement of the protoplasm, is plotted against time, an ordinarily smooth undulating curve is obtained. But frequently slight fluctuations of various kinds are obtained in the curves. These irregularities take the form of changes in amplitude with waxing and waning periods, of acute crest and flat trough, of a slight concavity following each crest and trough, etc. All variations occur in accordance with a rhythmic pattern.

A physiologist viewing the irregularities in the curves would attribute them to irregularities in protoplasmic behavior of which there are an ample number, but a physicist would see in the periodic recurrence of the fluctuations an example of interference such as is found in wave motion wherever studied (in optics, acoustics, and mechanics). If the curve of a protoplasmic wave motion showing interference phenomena is analyzed it will be found to be the resultant of two or more superimposed curves which differ in amplitude, or wave length. The situation is precisely the same as in acoustics, where two simple tones, the frequencies of which differ slightly, produce beats when sounded together. By compounding three specific curves of different wave lengths into a single harmonic curve, Kamiya (40) was able to produce exact counterparts of the experimental protoplasmic curve. The "abnormal" curves were therefore very normal ones, merely resultants of two or more pulsations of different amplitudes and phases. Three of these component rhythms, if they were not themselves resultants, were observed in the original moving pictures made of the rhythmic pulsations of slime molds (82).

The foregoing harmonic analysis of vital rhythms by Kamiya (40) is in itself commendable research work. But I wish to carry the conclusions still further. There is present in all protoplasm the potential capacity to pulsate rhythmically; when nature needs to use this capacity to its fullest extent, as in heart muscle, or occasionally, as in intestine muscle, it is available.



A second deduction which follows from the work on protoplasmic rhythm applies more specially to slime molds and other coenocytic forms among *Protista*. A multinucleate, noncellular plasmodium should be regarded as tissue, for, though devoid of walls and therefore of cells, it is nevertheless not a homogeneous mass but a protoplasmic system permitting the coexistence of many independent yet synchronized reactions such as rhythms, and this, as independent yet co-ordinated centers of activity, is tissue.

#### H. ANESTHESIA

The justification of a discussion on anesthesia in this review lies in the fact that the interpretation here given, essentially that of Claude Bernard, is a physical one. Before presenting it there are two questions to be disposed of, or, if that cannot be done, at least mentioned. One of these is the applicability of the term anesthesia to lower forms of life. To the biologist no such question exists; but to the medical man it is apparently quite impossible to anesthetize a protozoon, possibly because the protozoon lacks a central nervous system, or a "soul," or the capacity to suffer pain. The most remarkable explanation among the several given me is that in using the word anesthesia the biologist elevates himself to the status of a medical man. The reader may judge for himself which of these various reasons is justified, if any. I might remind him that the protoplasm of a slime mold, which is near the bottom of the plant and animal kingdom, is as much alive as is the protoplasm of our brain, and to this extent at least is itself a nervous system.

The second question is the applicability of a theory of anesthesia, based on the behavior of one type of organism, to all forms of life. That is a question which confronts us at every turn in our biological and medical research. It is a question which can be answered in part by experiment, but it is also in part a matter of philosophical outlook which rests on a belief or disbelief in the oneness of nature. We meet with it in all branches of thought, in the common origin of life, in the electromagnetic spectrum where what appear to be strikingly different forms of energy are but degrees of the same thing, in the identical patterns on which the smallest, atomic, and the largest, celestial, worlds are built. There is a strong modern tendency away from this type of thinking in biology and medicine due to overspecialization. We see it in a tendency to split species and split these again and again; and now we are confronted with it in the unwillingness of some investigators to recognize any general theory of anesthesia, preferring to re-

gard each genus—we hope it is not each individual—and each anesthetic agent as presenting a wholly new situation to be considered as a special case apart from all others. Fortunately, this trend away from general natural laws, from oneness in the physiological as well as the physical world, occasionally meets with convincing contradiction.

Without entering into an historical review of theories on anesthesia, I should like to turn directly to that theory which I advocate, being a re-interpretation of Claude Bernard's idea, for which I have the experimental evidence he lacked. The theory of anesthesia now generally accepted is that of loss of permeability control due to solubility of the fatty coating on cells by anesthetic agents which are usually fat solvents. But the best anesthetic agent for low forms of life is carbon dioxide and carbonic acid is not a fat solvent. Kurt Meyer, in a publication not yet available to me, gives further support to the lipide theory of anesthesia by stating that there is no parallelism between the effectiveness of an anesthetic agent and its adsorption, or its solubility in proteins, but that there is a perfect correlation between the efficiency of the anesthetic agent and its affinity for lipides.

Claude Bernard said that anesthesia is due to the coagulation of protoplasm. Because coagulated proteins are usually irreversible, and irreversibility means death, the theory was soon discarded. Numerous biologists have observed that the protoplasm of cells subjected to anesthetic agents ceases all visible activity. In the slime mold *Physarum* (85) the cessation of streaming on the application of an anesthetic agent is very striking because it is sudden, often instantaneous. It is due to a complete gelatinization of the protoplasm. Full recovery occurs when anesthesia has been successful. Carbon dioxide, cyclopropane, and chloroform accomplish the anesthesia of slime molds perfectly. Rapidly flowing protoplasm, when it is subjected to carbon dioxide, comes to a sudden stop through the process of gelatinization, and remains in this state until recovery takes place several minutes later.

The chief virtue of a purely physical interpretation of anesthesia lies in the fact that it fits all forms of anesthesia by whatever means produced. This no chemical hypothesis can do. The mechanism of anesthesia as here set forth is not to be expressed as the coagulation of protoplasm but as gelatinization, or thixotropic setting, for the latter two processes unlike the first are readily reversible. Identical results are obtained when anesthesia or the cessation of protoplasmic movement is accomplished by mechanical (88) or electrical shock.

That a state of anesthesia should exist when protoplasm has been gelatinized is evident from the fact that with an increase in viscosity there must be a decrease in metabolic activity which reaches a minimum at maximum viscosity, or at reversible gelatinization of the protoplasm. Not only is metabolism, taken as a whole, slowed down by gelatinization of the protoplasm, but either the gelatinization or the anesthetic agent itself undoubtedly interferes with enzyme activity.

### III. Chemical Constituents and Activities

#### A. PROTEINS

Sooner or later in a discussion of protoplasm one is likely to arrive at the question: is there in living matter one compound which represents the basic substance of life, or is protoplasm an association of many commonplace substances none of which taken alone is alive? The question cannot be answered. There may be one living substance which is beyond analysis because chemical methods destroy the specific vital qualities of protoplasm. What the test tube finally reveals has little resemblance to the substance which was once protoplasm. In any case it appears more likely that there is no one ingredient in protoplasm which is the ultimate living substance. This deduction leads to the conclusion that as all substances in protoplasm, taken alone, are nonliving, life appears only when all ingredients are present in specific proportions and properly assembled. Either view has essential elements of the other; thus, the belief that there is one ultimate living substance must take into consideration the importance of the extraneous matter in protoplasm; and in both assumptions the importance of structure or the proper assembling of parts is highly significant. If we may select one substance in protoplasm which is more significant than all others, it is certainly a protein, or a protein complex.

Oparin (64) lays emphasis on the part played by colloidal proteins in the origin of life. His sequence of events is: simple forms of organic matter, primary proteins, colloidal systems, primary organisms, and highly evolved organisms.

The conviction that the proteins are the all-important life-giving substances of protoplasm has led numerous investigators to assume that the basic proteins of living matter are enzymes. This view has been expressed by Alexander (1) and by Bergmann (10). The latter said that the hereditary substances in life are enzymes with the capability of synthesizing individual proteins by a predetermined sequence of specific reactions.

## B. ENZYMES

The degree of association between enzymes and protoplasm, or enzymes and proteins if protoplasm can only mean the entire heterogeneous mixture, undoubtedly varies from that of a free, water-soluble enzyme to an enzymic component of the protein or protoplasmic complex. Earlier biologists held to the view that vital enzymes and protoplasm are inseparable. Pasteur was of this opinion, certainly in the beginning of his career, when he regarded living cells as indispensable for alcoholic fermentation. This view, with its trace of vitalism, was upset by Büchner and Hahn, who found that cell-free extracts are capable of catalyzing the fermentation of sugar, a discovery which must rank with Wöhler's epoch-making synthesis of urea.

That protoplasm, in the last analysis, is an enzymic complex, is upheld by the extraordinary similarity between enzymes and protoplasm. Among nonliving substances none possesses properties so similar to those of protoplasm as do the enzymes. Both enzymes and living matter are destroyed by heat, by light, and by the same chemicals. Heat is destructive in each case at the same temperature (50°C.). Both enzymes and bacteria are highly resistant to heat when dry, and both are resistant to cold to an astonishing degree. Finally, no chemical is known that is injurious to bacteria yet without action on enzymes.

## C. ENERGY SOURCES

An attempt to distinguish between the living and the nonliving in a purely physical and chemical sense may be made on a structural and essentially static basis, or on an energy and therefore dynamic basis. Ordinarily such attempts are of the latter sort and center around concepts on the nature of the energy in living matter. All deductions on so theoretical a subject must at best assume a somewhat philosophical quality, but at that the conclusions reached can be kept within pure science by the exclusion of any concept based on an extramundane vital force. All forms of energy present in any system are, or should be, additive, but a supernatural vital energy cannot be included in the final sum, at least by any means known to modern science.

Energy sources in organisms are many, but most of them may be grouped under such familiar processes as respiration, fermentation, oxidation-reduction equilibria, hydrolysis, catalysis, and autocatalysis. These are all familiar reactions but the more we learn about certain of them the less clearly defined do they become, which is often true when a complete readjustment of basic concepts is in order.

### 1. *Hydrolysis*

The synthesis of proteins probably occurs through a coupled reaction. One member of such a couple cannot occur alone but may take place in the presence of another reaction which liberates the necessary energy; the two reactions occur simultaneously. Coupled reactions also control carbohydrate metabolism.

The energy-liberating reaction is not always known. One likely secondary reaction necessary for protein synthesis is the hydrolysis of other proteins, for synthesis and hydrolysis are constantly going on simultaneously in organisms. Northrop (63) states that the energy liberated by the hydrolysis of peptides in digestion could be returned to the protein peptide system and so accomplish the synthesis of proteins.

### 2. *Respiration*

Respiration is generally regarded as the source of energy necessary to support life. That this is true does not need verification in an advanced treatise, but one specific case may be cited. Borsook and Dubnoff (14) have found that the synthesis of hippuric acid from glycine and benzoic acid can occur only in the presence of intact liver cells. Extracts and minced tissues do not suffice, which leaves but the one conclusion, that the respiration of living tissue is the source of the energy.

That respiration, however defined, is the source of energy for life is widely recognized, but this rather obvious truth becomes obscured when respiration is delimited to a narrow class of energy-liberating reactions, as is done when it is defined as the oxidation of simple sugars with the aid of molecular oxygen. Viewed in a broader light, respiration is any protoplasmic reaction involving the release of energy for the support of life. Whether or not all forms of energy released in living matter, which in one way or another support life, are to be regarded as respiration is questionable. Modern physiological chemists (69) say not and so view respiration as including only these reactions which involve molecular oxygen. This means that the time-honored expression "anaerobic respiration" is out of order. So thinks Goddard (28), but not some other scientists (47,76,87,92).

When chemists found it necessary to broaden their interpretation of oxidation they did so. Oxidation need no longer involve oxygen. The same should be done for respiration, indeed, has long been done by biologists. When it is said of anaerobic organisms that they do not respire, then the physiology of life is viewed in a very narrow way. Respiration is the source of energy for life, and it may occur with or without oxygen. This is

the view which I (87) took of respiration, namely, that it is not merely a series of chemical reactions but a concept. The concept rests on the experimental fact that in all organisms there are energy-releasing reactions which support life, and these may or may not involve oxygen. The controversy is not a matter of terminology alone, or of good experimental work; it is a matter of interpretation and of broad thinking. Lundegårdh (47) views the problem in the broad biological sense when he states that the aerobic process predominates in natural life; anaerobic respiration is a relief when aerobic life is temporarily checked. Stiles (92) is of the same opinion.

For anaerobic respiration, modern physiological chemists (28,69) substitute the term fermentation. Historically, fermentation was not put on an oxygen-consuming or nonoxygen-consuming basis. The oxidation of alcohol to acetic acid was called fermentation by Pasteur, and it occurs with the help of gaseous oxygen. That anaerobic respiration may take place in the presence of oxygen (28) is of no significance; the important point is that molecular oxygen is not used. The whole matter is nicely settled by broadening the concept of respiration, just as the chemists did that of oxidation when they found the need for doing so.

Aside from the specific nature of respiration there is the question of its need during certain periods of rest in some organisms. No one questions the presence of respiration in some form in metabolically active protoplasm, but its need in certain forms of life when these are in a state of rest over long periods of time has been questioned (84). Seeds are known to live 200 years—spores are credited with longer rest periods but the evidence is not reliable. The sclerotium of slime molds is a hard, brittle sheet of protoplasm, as dry as paper, yet life continues. That respiration goes on in such forms of life at such times is usually assumed, but actually this is rather unlikely. The decomposition of matter in organisms which are not at the same time synthesizing organic substances would be a tremendous, dangerous, and wholly unnecessary waste, especially in organisms which rest over long periods of time. The assumption that respiration continues in an old but still viable seed or sclerotium is comparable to the assumption of the chemists that a catalyst accelerates or retards a reaction, but never initiates it. The two assumptions were made because biologists and chemists did not dare make the other assumption, that catalysts and protoplasm can initiate a reaction; but this assumption can be made.

It has of late been shown that protoplasm may be frozen and yet remain alive. Heretofore this was not thought possible, but it seems that although slow freezing results in death, rapid freezing by plunging into

liquid air leaves the tissue alive. It is inconceivable that respiration should continue in frozen protoplasm.

To say that respiration may be temporarily absent from living matter is a revolutionary statement. Whether or not it is true cannot be stated with finality. However, the important point is not whether respiration is present or absent in dormant protoplasm, but that there is no need for it. There is no need for it because respiration is not the distinguishing quality of life. I shall later point out that structural organization characterizes living matter, and as long as it is present there is no necessity for respiration or any other similar vital process to be active in resting protoplasm (84).

That a specific arrangement of parts is the essential attribute of living material is a purely hypothetical deduction and in no way alters the fact that respiration still remains the source of energy for metabolic processes in active protoplasm.

### 3. Autocatalysis

In a discussion on energy relationships in living matter, autocatalysis is frequently brought in as a specific and distinguishing property of protoplasm. But there is considerable difficulty in distinguishing the living from the nonliving on such a basis. On first thought, vital reactions, especially such significant ones as photosynthesis and respiration, would appear to be excellent examples of autocatalysis, for they are characteristic of life. Actually, however, neither photosynthesis nor respiration is autocatalytic. Autocatalysis would, therefore, appear to be less a distinguishing characteristic of protoplasm than ordinarily presumed, and much has been made of it. However, if autocatalytic reactions are those in which more of the catalyst is formed—such reactions have a typical S-shaped curve—then protoplasm in its entirety is certainly an autocatalyst. The multiplication of bacteria is said to be autocatalytic in form but not in fact, in form because more of the catalyst, protoplasm, is produced during the reaction, but not in fact because external energy, as food, is added. It seems that no purely autocatalytic reaction requires external energy. These truths, if they be such, bring us to anomalous situations, for we have set up two independent criteria of vital processes and possibly also of autocatalytic reactions: the quality of self-synthesis, and the question whether the energy term appears on the right-hand side of the equation (as when pepsin is autocatalytically formed from pepsinogen, 63), or on the left-hand side (as in the case of the growth of bacteria). The latter criterion is the one

used in classifying such unknown reactions as the formation of a virus. The difficulty here, however, is that no one yet knows where the energy comes from, nor will they know until the virus has been produced in a test tube.

And so we find that the two ways in which life and life-like reactions may be distinguished are quite independent of each other. Self-synthesis is one basis of classification, and self-sufficiency is the other. On the latter basis, there are two groups of reactions, those which will go by themselves and do not require the addition of energy, and those which do require the addition of energy. Autocatalytic reactions are distinguished on the first basis; they, theoretically at least, may or may not require energy.

The foregoing concepts require further elucidation, principally so that we may escape such anomalous situations as those which lead to the interpretation of growth and reproduction as autocatalytic, while at the same time admitting the need of an external energy supply. The two criteria of life, or catalysis, are not at all incompatible, but we cannot regard the multiplication of bacteria as autocatalytic and admit the need of external energy, and at the same time say that autocatalytic reactions not only involve self-synthesis but are also self-sufficient, for no autocatalytic reaction which requires external energy is known.

#### IV. Living vs. Nonliving

It may appear to some that we are now launched on a rather futile discussion, but actually the attempt to distinguish between living and nonliving has a sound biological basis. Hereditary qualities remain fairly constant; therefore, the substances by which they are transmitted must possess some quality, chemical or physical, which remains constant and is in some way highly specific.

Before attempting to interpret protoplasmic continuity in terms of structure, there may be paths of escape from a wholly materialistic view. Mind and psychic phenomena are often resorted to as final and conclusive proof that the living and the nonliving are of two worlds. But mind is not a prerequisite of life, for plants live.

The second law of thermodynamics is said not to apply to living matter in that nonliving systems ultimately attain a state of equilibrium whereas metabolically active protoplasm never does, but dormant protoplasm may.

And so we come at least to a possible answer to a question which might appear to be unanswerable. Just as continuity in inheritance depends upon structural continuity so does life itself. Living matter may owe its unique



quality to a specific structure, to a distinct arrangement of its parts. If this is true, then the transition from the nonliving to the living, acquired through specificity in structure, is a distinct step, and the transition, therefore, is not wholly continuous (84).

In order that the reader, as well as the author, may be left with his feet well planted on the ground, let us return to the measurable physical properties of protoplasm and point out that there is an extremely satisfying feature of recent work on protoplasm: the work of medical and biological scientists—Bensley (9), Scarth (72), Pfeiffer (68), Schmidt (74), Kamiya (40), Pease (66), Norris (59), and Northen (61)—and the work of the physical chemists—Astbury (4), Freundlich (23), Meyer (54), Mark (50), Treitel (95), and Treloar (96), are mutually supporting and lead to the same conclusion, that the physical properties of protoplasm and of nonliving organic systems (properties such as double refraction, elasticity, and the coexistence of rigidity and fluidity) point to structural continuity in protoplasm, accomplished through the interlocking of lateral bonds of molecular chains and molecular aggregates (51,56,83).

Pauling (65) expresses the foregoing conclusion in other words. He says that a specific type of molecular structure may be the basis of growth, the mechanism of reproduction of viruses and genes, the action of enzymes, the mechanism of the physiological activity of drugs, hormones, and vitamins, and the structure and action of nerve and brain tissue. To these activities of tissues determined by molecular structure, we may add muscular action (55,75), protoplasmic streaming (51,82), and certain metabolic activities of the cell (71) and the body as a whole (75).

It is not yet possible to show that the foregoing statements are true for such apparently complex and as yet little understood reactions as growth, reproduction, and nerve activity. But it is quite possible to explain such mechanical properties of protoplasm and its products as tensile strength, elasticity with fluidity, contractility, and imbibition in terms of molecular chains and the bonding of these fibers by cross ties.

## V. Structure

Scarth (72) has handled the coarser structure of protoplasm, or cell organization. He distinguishes five layers which, in general, occur in all cells. In most plant cells there is an additional layer, the outermost wall of cellulose. Within the cellulose wall there are, in order, the protoplasmic membrane or ectoplasm, the plasma gel or cortical endoplasm, the plasma-sol or liquid endoplasm, and the inner tonoplast or vacuolar membrane

surrounding the vacuolar fluid through which kinoplasmic strands thread their way, joining the outer membrane with the tonoplast.

Theories of the visible structure of protoplasm are classical, not fundamental. The statement does not imply that visible structure should be ignored; it plays a significant role in the metabolism of life, but it has no relation to basic structure of protoplasm. Advance in knowledge of the structure of living matter has been made in the ultramicroscopic not the microscopic field. Among newer concepts that of coacervates (34) has played a prominent role. According to Scarth (72), structural units of a coacervate are attracted by electrostatic forces and repelled by forces of solvation.

The structure which lies beyond the limit of microscopic visibility may be treated as a problem in chemistry or as a problem in physics. The former has been done by Bensley (8,9) and his students with the discovery of such possibly significant substances as the "structural protein ellipsin." Later Bensley reported the discovery of a sodium-chloride-soluble component of ellipsin, a highly viscous substance with pronounced elastic properties containing discrete fibers of great length. This he called plasmotin, regarding it as the structural foundation of tissue very much as is myosin in muscle.

Nature uses two types of proteins, linear ones such as myosin and globular ones such as albumin and globulin. The chain proteins are structural units used where strength is required; the spherical proteins are nutritional ones which, because of their shape, are readily moved about.

Though the protein chain has long been known, it was the cellulose fiber which first led to a sound conception of the fibrous structure of protoplasm and organic matter of high molecular weight. Meyer (53) and Mark (50) have led in this field. That the coarser structure of natural cellulose is fibrous is nicely shown by dissection work of the cell wall by Hock (36). The coarser visible cellulosic threads are made of long and tenuous fibrillae, and these in turn of linear molecules. Work on the proteins soon replaced that on cellulose in its bearing on the problem of protoplasmic structure.

The arrangement of the molecular fibers in mass has been the subject of much speculation, ranging from the early suggestion of Naegeli that the molecules aggregate into colloidal particles or micelles, to the modern concept of Astbury (2-4) that the molecular chains lie in parallel alignment so as to form grids. The parallel fibers are joined by lateral bonds the character of which has been the subject of much interesting speculation (49). Side chains which make lateral connections between molecules have long been recognized in stereochemistry.

In a chemical cycle, as in muscle, during which accessory molecules go in and out of combination with side chains, the protein main chains take up a cycle of configurations. In all such dynamic systems, of which protoplasm is one, there is a constant shifting of ties between the structural units; the loose union permitting this is very probably a hydrogen bond.

The new physicochemical concept of resonance will certainly find application to such biological problems as those presented here.

The biologist is confronted with many qualities of living matter which will remain purely physiological problems for some time to come, but the list of properties which are now fully or partially capable of purely physical or chemical analysis is a long one. It includes elasticity, tensile strength, contractility, thixotropy, gelation, fluidity, non-Newtonian behavior, streaming, amoeboid movement, structural continuity, birefringence, symmetry, asymmetry, spirality, liquid crystallinity, and selectivity.

It is truly an encouraging sign in the progress of science that properties of protoplasm such as contractility and structural organization, which heretofore were so little understood, can now be interpreted in terms of folded polypeptide fibers, interlocking side chains, hydrogen bonds, and asymmetry of the carbon atom.

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# RECENT VIEWS ON ASYMMETRIC SYNTHESIS AND RELATED PROCESSES

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## I. Introduction

It is some ten years since the subject of asymmetric synthesis and its bearing on the selective production of optically active compounds in nature has been fully reviewed. It was surveyed by McKenzie (82) in 1932; its relationship to the phenomenon of asymmetric induction was discussed in some detail by the present author (123) in 1933; and, finally, the posi-

tion up to 1935, with particular reference to the biochemical problem, was summed up with admirable brevity and succinctness by McKenzie (83).

In view of these surveys, the present résumé will not take the form of a detailed consideration of all aspects of the topic, for it is fair to say that no really fundamental advances have been made during the past ten years or so, though many results have been recorded which have filled relatively minor gaps in our knowledge, and have confirmed or refuted a number of doubtful earlier observations. There has, however, been a gradual crystallizing of views which permits us to correlate and systematize a number of different phenomena closely allied to asymmetric synthesis; and it is felt that a very useful purpose can be served at this stage by summarizing these recent views. Topics which have already been adequately reviewed will be dealt with only briefly, and attention will be given primarily to new results and some more recent points of view on earlier results.

## II. Asymmetric Induction, Transformation, and Synthesis

The conception of asymmetric synthesis has hitherto been based on Marckwald's classical definition (99):

*"Asymmetrische Synthesen sind solche, welche aus symmetrisch konstituierten Verbindungen unter intermediärer Benutzung optisch-aktiver Stoffe, aber unter Vermeidung jedes analytischen Vorganges, optisch-aktive Substanzen erzeugen."*

At the time this was formulated by Marckwald there was very little chance of confusion in its interpretation, though it is ironical that his own so-called asymmetric synthesis of valeric acid has subsequently been rather widely criticized and, as will be shown later (page 99), probably belongs to a category different from that envisaged by Marckwald himself. There were, however, a number of writers (149) who interpreted his definition rather loosely; and further confusion in thought and nomenclature has not unnaturally arisen owing to a variety of results reported during the past ten years or so, none of them complying strictly with the definition, yet all obviously closely connected with asymmetric synthesis. In an attempt to devise a systematic nomenclature based on first principles, we shall first of all consider the allied conceptions of asymmetric induction and asymmetric transformation, and their bearing upon asymmetric synthesis of classical type.

### A. ASYMMETRIC INDUCTION

Asymmetric induction is a conception of fundamental significance, which keeps recurring throughout the literature of stereochemistry, yet

which has persistently eluded the efforts of its sponsors to give it a solid experimental foundation unassailable by its many critics. It has been defined by Kortüm (61) as:

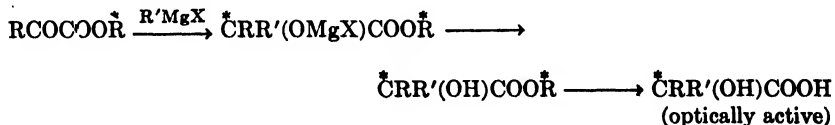
“... die Wirkung einer Kraft, die von asymmetrischen Molekülen ausgehend die Konfiguration veränderlicher Moleküle derart beeinflusst dass diese aus einer ursprünglich symmetrischen in eine asymmetrische Form übergehen.”

An effect of this type is obviously *intermolecular*, but it will be shown below that phenomena are also known which exemplify what may be considered as *intramolecular* asymmetric inductions.

Considerable use had been made of this conception by Erlenmeyer (36), who published many papers in which evidence was adduced that certain unsaturated groupings, notably C:C and C:O, containing no dissymmetric carbon atom, could assume a state of “induced” dissymmetry under the influence of adjacent optically active systems. This work has frequently been adversely criticized (33,35), and it is perhaps not unfair to say that Erlenmeyer's alleged optically active benzaldehyde and cinnamic acid cannot be regarded as having a real existence. At the most, they can only be regarded as assuming an exceedingly labile and transient form.

It is not always easy to use the terms *asymmetric* and *dissymmetric* with strict logical discrimination. Throughout this résumé, *dissymmetric* should be taken as applying to single individual molecules or groups, and *asymmetric* to an aggregate of dissymmetric molecules and to processes involving the preparation or decomposition of such aggregates. This nomenclature has also been used by Gause (43).

Later, McKenzie and co-workers (87,89,90,123) tentatively applied the conception to the mechanism of asymmetric syntheses of the following type, where  $\dot{R}$  is an optically active radical such as (–)-menthyl, and  $\dot{C}$  is a dissymmetric carbon atom.



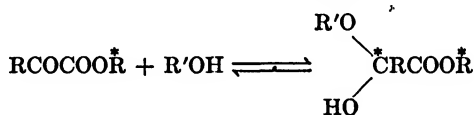
It was suggested that such a reaction involved the following stages: (a) Assumption by the  $\alpha$ -ketonic group of a novel type of dissymmetric configuration under the directing or inducing influence of the group  $\dot{R}$ , one “diastereoisomeride” preponderating over the other. (b) Fixation of



this labile configuration by the action of a Grignard reagent,  $R'MgX$ .

(c) Total elimination of the directing radical  $\dot{R}$  by saponification, thus revealing the optical activity of the resulting acid,  $CRR'(OH)COOH$ , one antipode preponderating over the other.

The evidence for the inclusion of stage *a* in the sequence was of two types. Roger and Ritchie (125) observed that optically active carboxylic esters,  $RCOOR^*$ , showed simple rotatory dispersion, contrasting markedly with the complex or anomalous dispersion of the corresponding  $\alpha$ -ketonic esters,  $RCOCOOR^*$ ; they attributed this to the existence in the ketonic molecule of two centers of dissymmetry—one fixed, the other induced and labile. Similar optical evidence for the existence of induced asymmetry has been summarized by Lowry (77). Further, McKenzie and Mitchell (87) and McKenzie and Ritchie (89,90) had already noted that alcoholic solutions of optically active  $\alpha$ -ketonic esters showed mutarotation. While it was fully recognized that this might be due to the gradual establishment of a new dissymmetric carbon atom  $\dot{C}$  by hemiacetal formation:



an explanation on the basis of asymmetric induction was provisionally preferred by McKenzie and co-workers as being more likely to shed light on the following further observations. With (–)-menthyl pyruvate, mutarotation showed as a numerical *increase* in optical rotation; and asymmetric syntheses by means of Grignard reagents yielded *dextrorotatory* substituted glycolic acids. On the other hand, with (–)-menthyl benzoylformate, mutarotation showed as a numerical *decrease*; asymmetric syntheses here led to *levorotatory* acids. This suggests a mechanism in which the induced asymmetry of the two types of  $\alpha$ -ketonic esters (opposite in sense, perhaps owing to the very powerfully negative nature of the phenyl group as contrasted with the methyl group) is reflected by mutarotation in opposite senses, and also by its fixation by Grignard reagents to give two oppositely rotating series of substituted glycolic acids. Out of some thirty asymmetric syntheses of this type only four or five exceptions to this relationship were noted. With some caution, therefore, the hypothesis that the first stage in these asymmetric syntheses is, in fact, an asymmetric induction was provisionally adopted as a useful incentive to further research.

Subsequently, Jamison and Turner (53) have investigated the kinetics of the above type of mutarotation, and conclude that there is so far no evidence that the mutarotation observed with an alcoholic solution of (—)-menthyl benzoylformate is due to anything more complex than simple solvation. Clearly, until mutarotation is observed in a solvent which excludes the possibility of hemiacetal or other complex formation, it cannot be admitted unreservedly as evidence of asymmetric induction.

Among those who have criticized the view that the mutarotation is due to asymmetric induction are Tiffeneau and his co-workers (139a). Instead of assuming that in the above type of asymmetric synthesis the  $\alpha$ -carbonyl group adopts a dissymmetric configuration prior to attack by the Grignard reagent, they prefer the view that the two bonds of the  $C=O$  group are attacked differentially by  $R'MgX$  when under the influence of an optically active system elsewhere in the molecule. They have produced evidence that, when the directing system and the carbonyl group are immediately adjacent, only one of the  $C=O$  bonds is ruptured, so that the new dissymmetric carbon atom adopts one exclusive configuration only. When, however, there is an intervening carbon atom, the effect appears not to be completely unilateral, as discussed more fully on page 89. Tiffeneau visualizes one of the  $C=O$  bonds as being attacked in a greater number of molecules than the other, leading to a new asymmetric system in which one configuration preponderates over, but does not exclude, its antipode. He makes the further suggestion, not as yet experimentally verified, that the effect may become progressively less and less unilateral as the number of atoms in the intervening chain is increased: and he attributes the relatively low preponderance of one antipode over the other in McKenzie's asymmetric syntheses to the separation of the  $\alpha$ -carbonyl group from the directing system by the  $-COO-$  portion of the ester group.

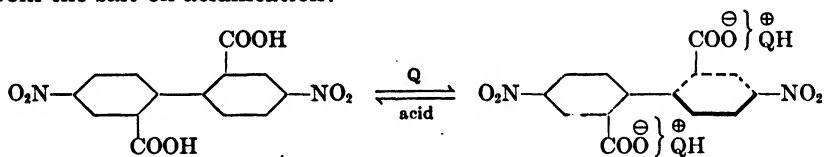
The two points of view are essentially qualitative, and may fairly be regarded as complementary rather than directly opposed.

## B. FIRST-ORDER ASYMMETRIC TRANSFORMATION

The literature contains many references to widely different molecular systems in which a fixed and stable center of dissymmetry has apparently influenced in a one-sided manner an adjacent center of dissymmetry of a more labile but classical type. Kuhn (66) attempted to correlate systematically a number of these systems by introducing the conception of asymmetric transformation. He subdivided this into "asymmetrische Umlagerung erster Art" and "asymmetrische Umlagerung zweiter Art." These

terms have been rendered into English in the literature as *first-order* and *second-order asymmetric transformations*. The translation of "Art" as "order" is perhaps not altogether happy, for there is a possibility of confusion with the terms "first order" and "second order" as applied to reaction kinetics. Jamison (51) has recently published an excellent critical résumé of present views on this topic, to which the reader is referred for fuller details; and Mills (103) has also discussed certain aspects of the problem. A short discussion of the main examples of Kuhn's two categories must suffice here.

Kuhn and Albrecht (67) noted that, when 4,4'-dinitrodiphenic acid is converted to its quinine (Q) salt, the latter is unexpectedly and strongly dextrorotatory; they attributed this to the adoption by the biphenyl skeleton of a dissymmetric configuration induced by the optically active alkaloid, and persisting for only so long as the salt exists. This can only be inferred and not proved directly, for only an inactive acid can be liberated from the salt on acidification:



Similarly, Pfeiffer and Quehl (119) found that addition of  $\alpha$ -phenanthroline to solutions of zinc camphorsulfonate causes a marked and unexpected diminution in the optical activity of the latter; this has been attributed to the one-sided formation of  $[\text{Zn}(\text{phen})_3]^{++}$  ions (which possess a dissymmetric octahedral configuration) under the asymmetric inductive influence of the sulfonate. Once again, however, direct and positive confirmation of this explanation is lacking, since only inactive  $[\text{Zn}(\text{phen})_3]\text{Br}_2$  could be isolated on treatment with potassium bromide.

Assuming for the moment the correctness of Kuhn's explanation of these and similar phenomena, we arrive at the conception of *first-order asymmetric transformation*—the activation of a configurationally labile racemate in solution by addition of an optically stable (+) or (−) compound which combines with the racemate to form a pair of diastereoisomerides, in unequal amounts at equilibrium. In favorable cases, slow establishment of equilibrium might show itself by mutarotation. Lesslie and Turner (72) originally claimed to have observed this for solutions of quinine diphenate in chloroform, though later Lesslie, Turner, and Winton (73) re-examined and withdrew this claim.

Jamison (51) points out that the essential condition for a first-order transformation is the reality of the diastereoisomerides *in solution*. If this is so, one would hardly expect the phenomenon to occur with diastereoisomeric salts in ionizing solvents such as water or even, in the case of quaternary ammonium salts, in solvents such as chloroform. McKenzie and Wood (93) have concluded that the effect recorded by Kuhn and Albrecht (67) may possibly have an alternative explanation.

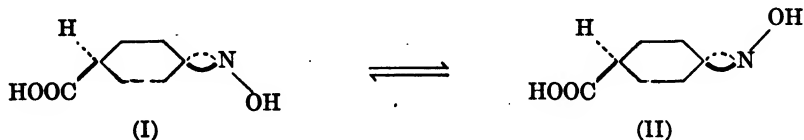
### C. SECOND-ORDER ASYMMETRIC TRANSFORMATION

Suppose, now, that the optically labile system in such a pair of diastereoisomerides possesses a fair degree of stability, though configurational inversion is still possible. What happens can best be understood by considering a few specific cases in which optical lability is due to a different type of cause in each case.

(a) **Optical Lability Due to *cis-trans* Isomerism.** Mills and Bain (104) noted that concentration of an aqueous solution of the quinine salt of oximinocyclohexane-4-carboxylic acid (which can exist in the antipodal forms I and II below) yields 80% of the quinine (–) salt. They suggested that:

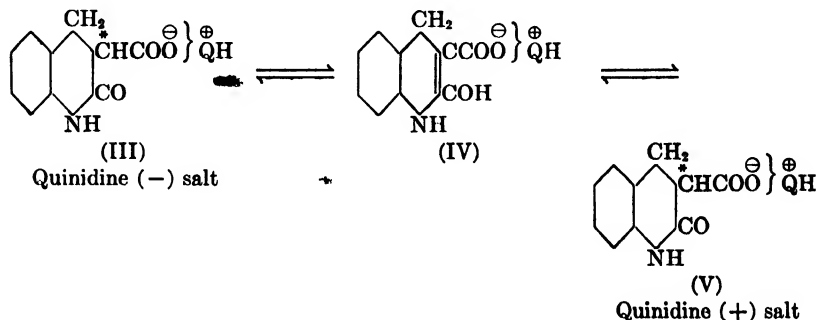
“... of the two diastereoisomeric quinine salts, that of the [(–) acid] must be the less soluble in water, and thus crystallizes first from an aqueous solution containing equal amounts of the two salts. The excess of [quinine (+) salt] thereby left in solution, however, racemises very readily, so that in spite of the removal of the [quinine (–) salt] an approximate equality is maintained between the quantities of the two salts in solution.”

The two salts are, of course, ionized in solution, and there are not in reality two diastereoisomeric salts until the crystallization point is reached. The optical lability of the acid is due to the relatively easy interconversion of the antipodal *cis* and *trans* oximes I and II, though the small energy barrier opposing such interconversion is sufficient to permit the (–) acid to be isolated on acidification of the brucine (–) salt:

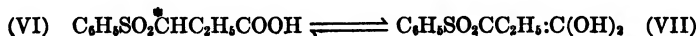


(b) **Optical Lability Due to Tautomeric Equilibrium.** Leuchs and Wutke (75) and Leuchs (74) have recorded two transformations with a different mechanism but a similar result. For example, in attempting to

resolve *dl*-hydrocarbostyryl-3-carboxylic acid by quinidine (Q), Leuchs found the *dl* acid to be progressively and completely activated to the (+) form. Of the two diastereoisomeric salts III and V, the latter is the more sparingly soluble and crystallizes first. Since the acid ion is optically labile due to a keto-enol equilibrium involving the dissymmetric center  $\overset{*}{C}$ , the equilibrium pictured below is displaced continuously from left to right, via the enol (IV), as V crystallizes out:

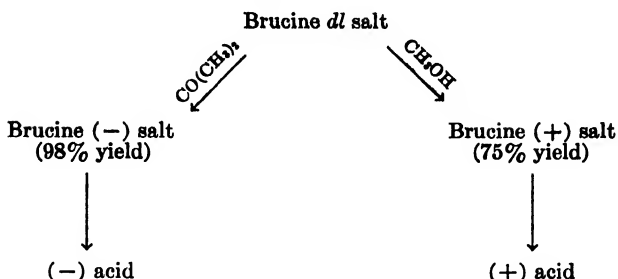


A similar effect has been noted by Ashley and Shriner (5) with *dl*- $\alpha$ -phenylsulfone-*n*-butyric acid (VI), where only the brucine (-) salt crystallizes from acetone. Optical lability was attributed to an equilibrium between VI and VII, involving the dissymmetric center  $\overset{*}{C}$ , though once again the (-) acid is sufficiently stable to be isolated on acidification of the alkaloidal salt:

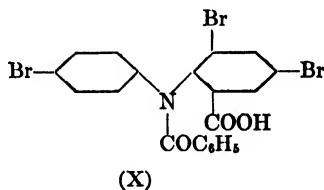
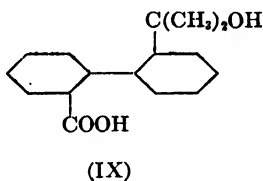
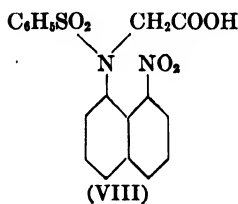


(c) **Optical Lability Due to Systems Showing Restricted Rotation.** Molecules like suitably substituted biphenyls, etc., can show dissymmetry owing to restricted rotation within the molecule, but are nevertheless optically labile due to the fact that, given sufficient energy, they can overcome the energy barrier opposing passage through the flat symmetrical configuration to yield the antipodal form; and they are in many ways ideally suited to the investigation of problems of asymmetric transformation.

Thus, Mills and Elliott (105) found that on crystallizing the brucine salt of *N*-benzenesulfonyl-8-nitro-1-naphthylglycine (VIII) from two different solvents, acetone and methyl alcohol, an asymmetric transformation can be effected at will in two opposite senses. In effect, a novel type of resolution is achieved in this special case:



Corbellini and Angeletti (24) and Jamison and Turner (54) investigated the activation of 2'-( $\alpha$ -hydroxyisopropyl)diphenyl-2-carboxylic acid (IX) by means of brucine, and noted the crystallization of the pure brucine (-) salt, from alcohol, in yields as high as 97.6%.



Jamison and Turner (52) have worked intensively on optical activation of various substituted *N*-benzoyldiphenylamines by means of alkaloïds and have achieved particularly interesting results with the acid, *N*-benzoyl-2,4,4'-tribromodiphenylamine-6-carboxylic acid (X). Conditions were here suitable for demonstrating the fairly marked temperature coefficient of the inversion process, for the acid in acetone solution is optically labile at room temperature, but stable at  $-15^\circ\text{C}$ . Crystallization of an equimolecular solution of the *dl* acid and cinchonidine from acetone at room temperature led to separation of pure cinchonidine (+) salt in almost 100% yield, a typical second-order asymmetric transformation. However, the same process at  $-15^\circ\text{C}$ . led to crystallization of cinchonidine (+) salt in only about 50% yield, and the residue on evaporation of the cold filtrate *in vacuo* was about two-thirds cinchonidine (-) salt—in other words, a typical resolution by the classical salt-formation method.

Analogous results have also been recorded in this field by Davidson and Turner (29), the labile acid here being benzylmalonoanilic acid,  $\text{C}_6\text{H}_5\text{CH}_2\text{CH}(\text{COOH})\text{CONHC}_6\text{H}_5$ , or one of its derivatives.

## D. BORDERLINE CASES OF ASYMMETRIC TRANSFORMATION

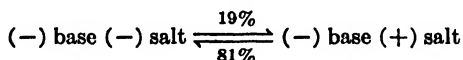
It is obvious from the above that there is no clear-cut distinction between first-order asymmetric transformations, second-order asymmetric transformations, and the process of resolution by salt formation. An acid so optically stable that it does not undergo configurative inversion within the accessible range of experimental conditions will show straightforward resolution; but an acid with a marked temperature coefficient of optical inversion might be made to show all three variations of the phenomenon under appropriate temperature conditions.

Kuhn himself recognized the possibility that borderline cases, neither "erster Art" nor "zweiter Art," might exist. Jamison and Turner (54) consider Kuhn's insistence—that an asymmetric transformation is of the second order only if the labile system shows a detectable optical activity after removal of the stable directing system—to be an immaterial and arbitrary distinction:

"Second order asymmetric transformation, in any case in which interconversion of diastereoisomerides is possible (first order transformation) and crystallisation can be induced, may be expected to be almost quantitatively realisable, and to give one diastereoisomeride in the optically pure crystalline condition."

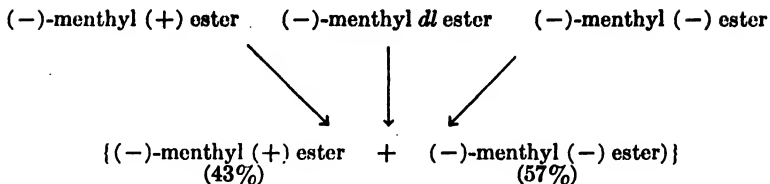
Jamison and Turner regard this as the essential criterion.

Read and McMath (122) prepared (–)-hydroxyhydrindaminechlorobromomethanesulfonate, and observed a marked mutarotation in acetone solution. The salt of the (+) base showed a corresponding mutarotation in the opposite sense. The authors calculated that the following equilibrium had been set up:



Despite intensive effort, however, they were unable to remove the base in such a way as to liberate an optically active acid, so that, in the narrow sense of Kuhn's original definition, this would have to be classed as a first-order transformation.

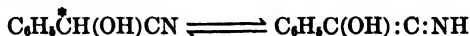
The phenomenon known as *asymmetric catalytic racemization* may also be considered as illustrating the possible existence of borderline cases. McKenzie and Smith (92) observed mutarotation when a trace of alcoholic potash is added to alcoholic solutions of (–)-menthyl *dl*-phenylchloroacetate (and similar esters), the final rotation corresponding to an equilibrium mixture which, as the following scheme shows, could be reached from three different starting points:



The optical lability of the phenylchloroacetyl group, due to tautomeric change involving the dissymmetric carbon atom, is so marked in the presence of alkali that separation of the stable system from the labile by saponification (for we are dealing here with an ester, not a salt) results in the formation of the racemic acid. None the less, it is not inconceivable that refinements in technic might some day remove any formal objection to classifying this phenomenon as a second-order asymmetric transformation; the same may apply to that recorded by Read and McMath (122).

Additional evidence that the degree of optical lability of the phenylchloroacetyl group lies within a rather critical range is the fact that McKenzie and Smith (91) found it possible to effect a partial separation or resolution of the diastereoisomeric forms of  $(-)$ -menthyl *dl*-phenylchloroacetate by repeatedly crystallizing the latter from ethyl alcohol.

Similar arguments apply to the asymmetric catalytic racemization of amygdalin, recorded by Smith (133). Amygdalin is the gentiobioside of  $(+)$ -mandelonitrile. The extreme optical lability of the latter is reflected in the great precautions which have to be taken to avoid its racemization, for the pure crystalline  $(+)$  nitrile is readily racemized by the presence of even a trace of water, probably by a mechanism of the type suggested by Fischer and Bergmann (40):



Here again, then, we see the artificial nature of Kuhn's original distinction between first- and second-order asymmetric transformations. A technic less refined than that employed in Smith's preparation of pure  $(+)$ -mandelonitrile (132) would exclude the possibility of proving directly the order of the transformation.

Of the above results, Jamison (51) remarks that:

"... the nature of their material and the fact that the activated group could not be isolated without isomeric change made this work of McKenzie and Smith unsuitable for the inception of a general theory [of asymmetric transformation]: with the theory established, their experimental work falls into place and lends it convincing support."

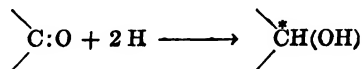


### E. ASYMMETRIC SYNTHESIS AS A PARTICULAR CASE OF ASYMMETRIC TRANSFORMATION

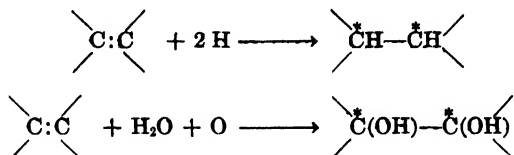
It will now be of interest to consider whether the classical examples of asymmetric synthesis can be fitted into the broad new generalization outlined above.

Unsaturated groupings such as C:C and C:O, when included in a molecular system containing an optically stable directing or inducing group, have already been considered tentatively to assume an induced dissymmetric configuration (page 67). The precise nature of this configuration is not postulated with certainty, but it may perhaps be due to a type of polar activation such as that discussed by Lowry and Owen (78), displacement of a pair of electrons away from one carbon atom toward the other (or toward the ketonic oxygen atom) eventually producing a semipolar double bond.

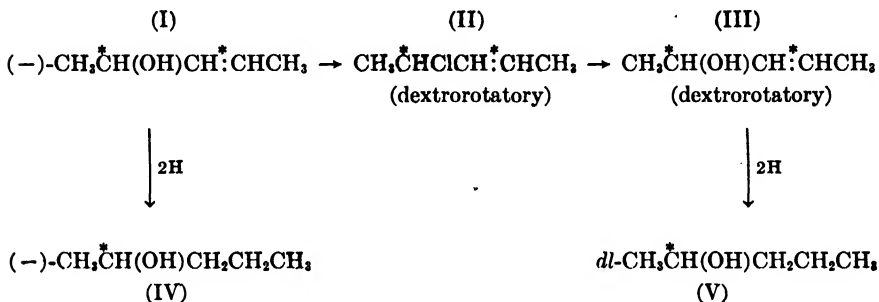
It would be expected that any such induced asymmetry in an aggregate of molecules would be too labile to survive removal of the stable inducing group. Its existence, therefore, cannot be demonstrated directly; but, if it indeed exists, its establishment might logically be classed as a first-order asymmetric transformation. The most obvious method of reducing the optical lability of the induced system to such an extent that a stable optically active product is produced after elimination of the stable directing or inducing group is to "lock" or "fix" its configuration by a suitable chemical reaction. Many classical asymmetric syntheses are known, mostly due to McKenzie and co-workers, which may be considered as examples of such a fixation. Thus, when optically active  $\alpha$ -ketonic esters form the starting point, the "induced"  $\alpha$ -carbonyl group becomes a fixed center of classical dissymmetry, either by reduction (80,86,88) or by a Grignard reaction (79,85,89,90,94):



Similarly, in optically active esters containing an olefinic group, the induced C:C group yields one center (or two) of classical dissymmetry, either by reduction (76,136,143) or by addition of two hydroxyl groups (95):

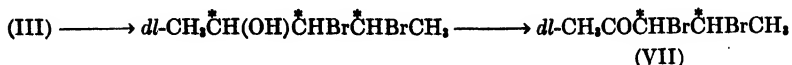
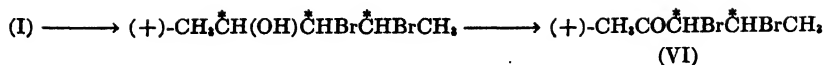


The conception of asymmetric induction as a first stage in the process of asymmetric synthesis has appeared to some workers as fundamentally necessary, but more recent results have thrown some emphasis on an alternative mechanism. Hills, Kenyon, and Phillips (47) have realized the following remarkable scheme experimentally:

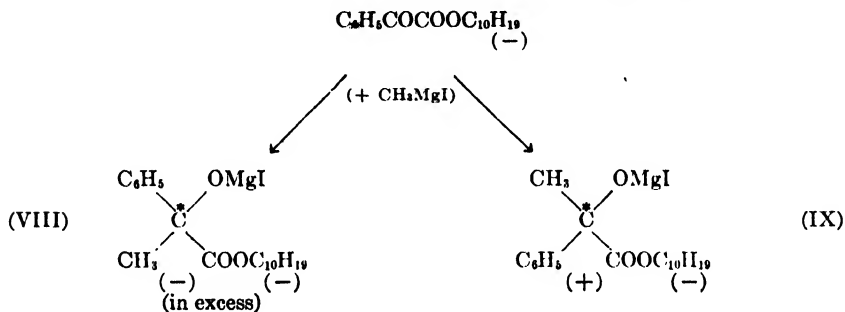


The pure (–) alcohol (I) yields, via the chloride (II), a dextrorotatory alcohol (III). Both I and III yield methyl-*n*-propylcarbinol on catalytic reduction; but, whereas IV is the optically pure (–)-carbinol, V is the *dl* form. Hills, Kenyon, and Phillips drew the very remarkable but not unreasonable conclusion that, in I, the olefinic system is in a state of induced asymmetry (a conclusion supported by the fact that the rotatory dispersion of I is anomalous, presumably owing to the optical contribution of the induced system), and that, in III, the optical activity is due solely to the residual induced asymmetry, the contribution from the fixed center having been destroyed by racemization or inversion during the two replacement reactions.

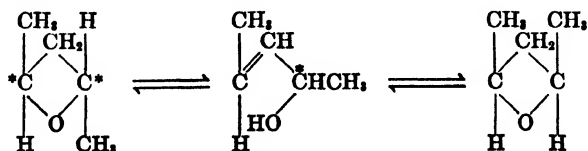
Assuming this to be so, Kenyon and Partridge (58) devised a novel experiment to test whether the induced asymmetry of an olefinic system, as indicated by its contribution to the optical activity of a substance, could, in fact, on fixation by an addition reaction cause an asymmetric synthesis. Starting from I and III, they successively brominated and oxidized according to the following scheme:



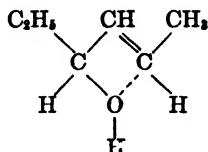
Since VII is optically inactive, despite the dextrorotation of III; Kenyon and Partridge considered that in the type of asymmetric synthesis effected by McKenzie it is not fixation of induced asymmetry by addition of RMgX which causes the addition to proceed one-sidedly, but simply a difference in the energies associated with the diastereoisomeric intermediates (VIII and IX). They suggested that collisions with the addendum likely to give rise to VIII are more favored than those giving rise to IX:



It is only fair to point out, however, that the idea that III owes its optical activity solely to persistence of induced asymmetry in the olefinic system, after racemization of the group  $\text{CH}_3\text{CH}(\text{OH})\text{—}$ , is a very revolutionary one, and requires full investigation before the ingenious experiment of Kenyon and Partridge is accepted at its face value. III may, of course, prove to be the first example of a substance owing its optical activity to induced asymmetry only; but it may, on the other hand, prove to have quite a different constitution from that suggested. For example, Hills, Kenyon, and Phillips mentioned but were inclined to reject the possibility that it existed in the following tautomeric forms:



Airs, Balfe, and Kenyon (3) later suggested for the analogous  $\gamma$ -methyl- $\alpha$ -ethylallyl alcohol a resonance structure of the following cyclic type:



No final choice between the two mechanisms is possible on the present evidence, though the experiments recorded by McKenzie and Christie (84), and discussed on page 96, cast some doubt upon the collision mechanism suggested by Kenyon and Partridge.

### III. Classification of Methods of Producing Optically Active from Optically Inactive Substances

On the basis of the foregoing discussion, we can now attempt a new and full classification of the various methods—some old, some very recent—of producing optically active from optically inactive substances. We must consider in particular the allocation of asymmetric synthesis, in the sense defined by Marckwald, to its correct place in the scheme; and we must also consider how many of the processes may operate in various biological syntheses and decompositions.

The classification rests on the recognition of the four broad categories: (A) resolution of optically stable racemates, (B) optically selective inversion of configuration in optically labile racemates, (C) optically selective synthesis of new centers of dissymmetry, and (D) optically selective inactivation of existing centers of dissymmetry.

The various known methods are tabulated below, under a tentative systematic nomenclature and with brief definitions. Each method is then considered in turn, with special mention of any recently published examples, and with notes on the possibility of its biological occurrence.

#### (A) *Resolution of optically stable racemates.*

- (1) *Mechanical resolution of a dl conglomerate.* Separation by hand of crystals exhibiting visible enantiomorphism.
- (2) *Resolution by inoculated crystallization.* Optically selective crystallization from supersaturated solution of a racemate after inoculation with unsymmetrical crystalline nuclei.

- (3) *Resolution by optically selective adsorption.* Optically selective adsorption of a racemate on a solid optically active adsorbent.
  - (4) *Resolution through stable diastereoisomerides.* Conversion of a racemate to a mixture of diastereoisomerides, separation of the latter by taking advantage of their different physical properties, and their subsequent suitable decomposition.
  - (5) *Interrupted diastereoisomeride formation.* Interrupted optically selective combination of a racemate with an optically active substance.
  - (6) *Interrupted diastereoisomeride decomposition.* Interrupted optically selective decomposition of a compound of a racemate with an optically active substance.
  - (7) *Interrupted asymmetric catalysis.* Interrupted optically selective reaction of a racemate with a symmetrical substance under the intermolecular influence of an optically active catalyst.
- (B) *Optically selective inversion of configuration in optically labile racemates.*
- (1) *Second-order asymmetric transformation.* Combination of an optically labile racemate with a stable, optically active substance in solution, crystallization under such conditions that the separation of the less soluble diastereoisomeride is more rapid than the interconversion of the labile antipodes of the original racemate, and subsequent removal of the added optically stable substance from the solid product.
- (C) *Optically selective synthesis of new centers of dissymmetry.*
- (1) *Absolute asymmetric synthesis.* Optically selective formation of dissymmetric molecules by the interaction of symmetrical molecules or groups under the influence of an unsymmetrical physical agency (e.g., circularly polarized light).
  - (2) *Asymmetric synthesis (eliminative).* Optically selective formation of new centers of stable dissymmetry under the intramolecular directing influence of an optically active grouping, the latter being subsequently eliminated from the product.
  - (3) *Internal asymmetric synthesis (noneliminative).* Optically selective formation of new centers of stable dissymmetry under the intramolecular directing influence of an optically active grouping, the latter being subsequently inactivated without actual elimination from the product.
  - (4) *Catalytic asymmetric synthesis.* Optically selective formation of new centers of stable dissymmetry under the intermolecular influence of an optically active catalyst.
- (D) *Optically selective inactivation of existing centers of dissymmetry.*
- (1) *Absolute asymmetric decomposition.* Optically selective decomposition of dissymmetric into symmetrical molecules under the conditions of C1.
  - (2) *Eliminative asymmetric decomposition.* Optically selective decomposition at existing centers of dissymmetry under the conditions of C2.
  - (3) *Noneliminative asymmetric decomposition.* Optically selective decomposition at existing centers of dissymmetry under the conditions of C3.
  - (4) *Catalytic asymmetric decomposition.* Optically selective decomposition at existing centers of dissymmetry under the conditions of C4.

#### IV. Detailed Consideration of the Foregoing Classification

It is not suggested that the foregoing classification avoids the problem of classifying awkward borderline cases. Certain of the categories defined may be regarded as limiting cases of a continuously graded series of phenomena; some of these will be mentioned in the discussion which follows. Furthermore, although practically all the results recorded in this field can be convincingly assigned to one of the categories now defined, there are still a few examples which can be assigned only provisionally, due either to the incompleteness of the published experimental data, or to our incomplete knowledge of the precise reaction mechanism.

##### A. RESOLUTION OF OPTICALLY STABLE RACEMATES

(A1) **Mechanical Resolution of a *dl* Conglomerate.** This is, of course, Pasteur's classical method, and calls for no special comment, except to emphasize its limited applicability.

(A2) **Resolution by Inoculated Crystallization.** This is exemplified by Purdie's resolution of lactic acid (121) by inoculation of a supersaturated aqueous solution of zinc ammonium *dl*-lactate with a crystal of zinc ammonium (+)- or (-)-lactate. Ostromisslensky (115) claimed that inoculation of a supersaturated solution of *dl*-asparagine with a crystal of glycine causes preferential deposition of *one* antipode only. This surprising result was attributed to the existence of undetected hemihedrim in the *crystals* of glycine, the *molecules* themselves being, of course, symmetrical. The work, however, requires careful repetition to confirm that the alleged resolution was not, in fact, due to some fortuitous cause such as the existence of optically active crystalline nuclei in the atmospheric dust of the laboratory, as suggested by Kipping and Pope to account for certain of their experimental results (59). If Ostromisslensky's result is confirmed, it exemplifies a mechanism which might be operative in certain biochemical processes.

(A3) **Resolution by Optically Selective Adsorption.** This is a relatively new technic, and one which may well have a counterpart in biological processes. Tsuchida, Kobayashi, and Nakamura (141) and Karagunis and Coumoulos (56) have reported the optically selective adsorption of certain racemic co-ordination compounds such as  $[\text{Co}(\text{en})_2]\text{Cl}_2$  and  $[\text{Co}(\text{C}_2\text{O}_4)_2]\text{K}_2$  on crystals of optically active quartz. Later, Henderson and Rule (45) achieved a very marked resolution of *dl-p*-phenylenebis-iminocamphor,  $\text{C}_{10}\text{H}_{16}:\text{NC}_6\text{H}_4\text{N}:\text{C}_{10}\text{H}_{16}$ , by a chromatographic technic

using crystalline lactose as the adsorbent. More recently, Martin and Kuhn (101) made an ingenious application of the principle to the resolution of *dl*-mandelic acid by optically selective adsorption on wool fibers. A similar result had previously been claimed by Porter and Ihrig (120); certain racemic dyestuffs were here adsorbed on wool. However, this last result has been adversely criticized by Brode and Adams (18), who could not confirm the effect reported.

The above work on optically selective adsorption has a close bearing on an earlier observation by Schwab and Rudolph (128a) that, when 2-butanol is catalytically dehydrated over active quartz, the unchanged alcohol is found to have developed an optical rotation. This is apparently due to an optically selective adsorption of (+) or (-) 2-butanol on the enantiomorphous crystals of the catalyst.

**(A4) Resolution through Stable Diastereoisomerides.** This is the best known and most widely applicable of the methods of resolution. Even so, one aspect of the inclusion under this heading of fractional crystallization of diastereoisomeric salts and esters calls for mention. If a racemic acid A is *completely* optically stable under the conditions of its resolution by an optically active base, say, (+)-B, then the diastereoisomerides, (+)-A (+)-B and (-)-A (+)-B, will be formed in solution in equal amounts, and fractional crystallization will not disturb this ratio when all solid and liquid phases are taken into account. But, if A is even slightly labile, crystallization may lead to partial transformation of the more soluble into the less soluble diastereoisomeride—a second-order asymmetric transformation. In many cases it may be difficult to decide whether the resolution of, say, a racemic acid by means of an alkaloid is the true limiting case, A4, with no configurational inversion whatever, or very slightly one-sided second-order transformation (page 84).

An unusual type of optical activation recorded by McKenzie (81) may be considered here. It was found that, when *dl*-tartaric acid is neutralized with one molecular proportion of caustic potash, and one molecular proportion of (-)-malic acid is added to the solution, a mixture of *dl* and (+) tartrates crystallizes out; a similar though less pronounced activating effect has more recently been observed with (+)-citramalic acid (2). While suggesting very tentatively that these effects might be considered examples of asymmetric induction, McKenzie at the same time pointed out that they might also be due to an unusual partial resolution of a racemic acid by an optically active acid, due to physical differences between a pair of diastereoisomeric double salts. The latter explanation is perhaps the more likely.

Methods of separating diastereoisomerides other than by fractional crystallization have been developed comparatively recently. Fractional distillation has been successfully applied by Bailey and Hass (7), and fractional distribution between immiscible solvent pairs by Shapiro and Newton (129). Fractional adsorption on symmetrical adsorbents such as silica gel and carbon has also been achieved, chromatographic methods with solutions having been applied by Jamison and Turner (55) and Hass, de Vries, and Jaffé (44), while Isom and Hunt (49) have noted fractional adsorption from the gaseous phase.

**(A5) and (A6) Interrupted Diastereoisomeride Formation and Decomposition.** These categories are exemplified by the fundamental work of Marckwald and McKenzie (100) on the esterification of *dl*-mandelic acid by (–)-menthol, and the saponification of (–)-menthyl *dl*-mandelate. Carried to completion, neither process shows resolution, but interruption before completion gives a form of partial resolution, owing to the different rates of formation and saponification of the diastereoisomeric (–)-menthyl mandelates. This is not, perhaps, a resolution in the ordinary sense of the term, though in suitable cases it would be possible to obtain, by saponification of the ester existing at any stage of interruption, an optically active acid opposite in sign to the free optically active acid.

**(A7) Interrupted Asymmetric Catalysis.** Not many reactions in this category, using catalysts of known constitution, have been recorded. The principal examples are to be found in a series of papers by Wegler (144,145), who observed, for example, that esterification of *dl*-phenylmethylcarbinol by acetic acid and formation of a urethan from *dl*- $\alpha$ -phenylethylamine and chloroformic ester proceeded asymmetrically in the presence of an alkaloidal catalyst such as brucine.

When we turn to the use of enzymic catalysts of unknown constitution, several striking and well-known examples fall into this category. The optically selective enzymic hydrolysis of certain esters of *dl* acids with symmetrical alcohols, or of *dl* alcohols with symmetrical acids, has been noted by several workers. Dakin (25), for example, found that liver lipase hydrolyzes ethyl *dl*-mandelate and benzyl *dl*-mandelate in a selective fashion; Neuberg, Wagner, and Jacobsohn (112) noted the similar effect of a vegetable phosphatase upon *dl*-bornyl dihydrogen orthophosphate. These reactions can be regarded as an extension of the principles postulated by Marckwald and McKenzie (100), for the selective enzymic hydrolysis of the antipodal esters could be demonstrated only by interrupting the process before completed hydrolysis had been achieved. Willstätter,



Kuhn, and Bamann (147), and many other workers, have recorded similar results with a variety of different esterases.

## B. OPTICALLY SELECTIVE INVERSION OF CONFIGURATION IN OPTICALLY LABILE RACEMATES

(B1) **Second-Order Asymmetric Transformation.** Enough examples of this class have been quoted previously to illustrate its general character, but a few elaborations of the argument should be considered at this point.

In the first place, it should be noted that such reactions have a most important bearing on optically selective biosynthesis. Kuhn (68) has pointed out that the deposition of pure amygdalin in plant tissues is not necessarily due to an optically specific enzyme. Amygdalin itself crystallizes readily from water, but, as Krieble (64) has shown, its diastereoisomeride, the gentiobioside of (—)-mandelonitrile, does not. If we bear in mind the marked optical lability of mandelonitrile, it is clear that this solubility difference may readily account, on the basis of second-order asymmetric transformation, for the storage of amygdalin, but not isoamygdalin, in plant cells. Kuhn described substances such as gentiobiose, which play a constant directive role of this kind, as "stereo-autonomic," and his arguments have been elaborated in a recent publication by Gause (43). It is quite clear that what is being described is a second-order asymmetric transformation.

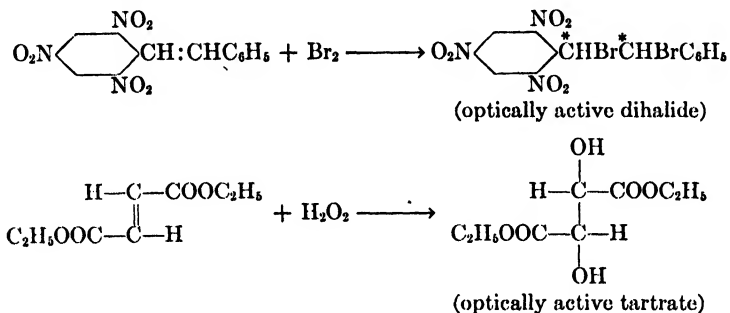
The occurrence of the gentiobioside of the (+) nitrile in the fruits of *Prunus laurocerasus*, and of the glucoside of the *dl* nitrile in the leaves of the same plant, is suggested by Kuhn to be most probably a reflection of greater solubility differences between the gentiobioside diastereoisomerides than between those of the glucoside. One is tempted to speculate whether a similar reason may underlie the occurrence of (+)- and (—)-pinene in different parts of one and the same plant, and the occurrence of both (—)- and *dl*-borneol in valerian oil (128).

## C. OPTICALLY SELECTIVE SYNTHESIS OF NEW CENTERS OF DISSYMMETRY

(C1) **Absolute Asymmetric Synthesis.** Several examples of absolute asymmetric synthesis have been recorded during the past ten years or so. In 1933, Karagunis and Drikos (57) brought about the optically selective photochemical union of halogens with certain triarylmethyl free radicals, CRR'R", by irradiation with circularly polarized light. The

optically active product was presumed to be a triarylmethyl halide; with the phenyl-*p*-xenyl- $\alpha$ -naphthylmethyl radical, it was found possible to reverse the sign of rotation by an alteration in the wave length of the irradiating beam.

Later, Davis and Heggie (31) and Davis and Ackermann (30) adopted a different approach. Circularly polarized light was employed to bring about the photochemical addition of halogens to 2,4,6-trinitrostilbene, whereby a feebly optically active 2,4,6-trinitrostilbene dihalide was obtained (31), and also to bring about photochemical hydroxylation of ethyl fumarate in the presence of hydrogen peroxide, which yielded a feebly optically active ethyl tartrate (30):



The above experiments deal with reactions in the liquid phase; but Betti and Lucchi (9) claimed to have effected absolute asymmetric syntheses in the gaseous phase. Addition of chlorine to propene and to butadiene, and of hydrogen chloride to 2-butene, yielded dextrorotatory products during irradiation with dextrorotatory polarized light, and levorotatory products with levorotatory polarized light.

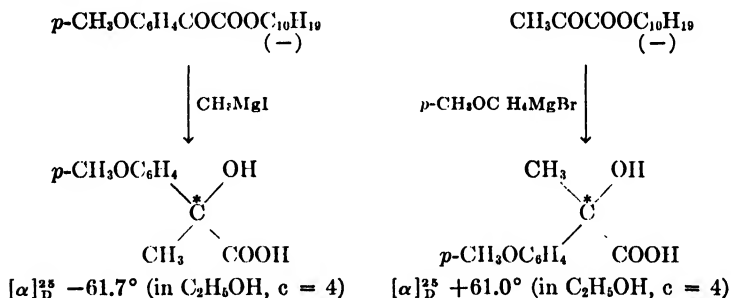
It is to be noted that in each experiment the maximum observed rotation was very small. The largest recorded angle was  $\pm 0.2^\circ$ , and in most of the experiments was of the order of only  $\pm 0.05^\circ$ , which indicates a very low order of optically selective efficiency even under the chosen favorable conditions of the laboratory.

**(C2) Asymmetric Synthesis (Eliminative).** This is the classical type of asymmetric synthesis, complying with Marckwald's definition.

It includes McKenzie's syntheses of optically active  $\overset{*}{\text{CHR}}(\text{OH})\text{COOH}$  (by the reduction of optically active  $\alpha$ -ketonic esters), of  $\overset{*}{\text{CRR}}'(\text{OH})\text{COOH}$  (by Grignard reactions with the same esters), and of tartaric acid (by hy-

droxylation of optically active fumarates by means of aqueous potassium permanganate), and also the synthesis of optically active  $\beta$ -phenyl-*n*-butyric acid by reduction of optically active esters of  $\beta$ -methylcinnamic acid. In each case, the optically active directing system is completely removed, by saponification, after fixation of the induced asymmetric system by an addition reaction.

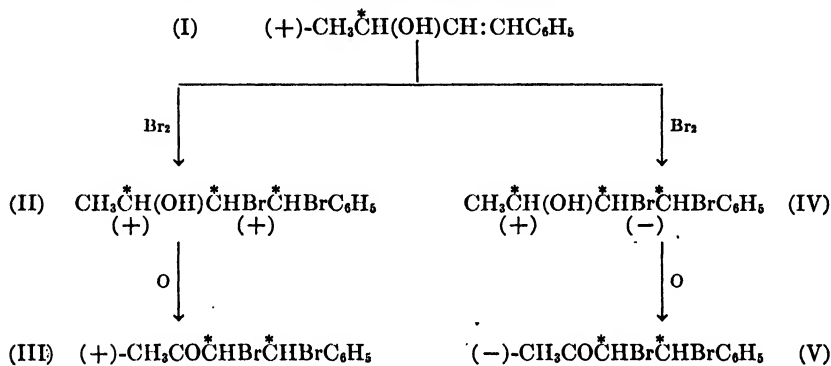
There is little fresh to be said under this heading, but it may be worth while emphasizing that the products from McKenzie's syntheses were far from being optically pure. In other words, the mechanism is only partially and not completely optically selective. In the majority of the syntheses, crystallization of the optically active product led to separation of *dl* acid, and the excess of one antipodal form remained in the mother liquor. In rare cases, however, the solubility relationships between antipodes and racemate were such that the excess of one antipodal form separated first in the optically pure state. Thus, McKenzie and Ritchie (90) effected the following pair of syntheses:



Christie, McKenzie, and Ritchie (21) subsequently confirmed the optical purity of the above anisylmethylglycolic acids by a resolution of the *dl* acid with morphine. A similar result was recorded by McKenzie and Christie (85), the action of methyl magnesium iodide on (–)-menthyl *p*-toluylformate leading to the isolation of crystalline (–)-methyl-*p*-tolylglycolic acid admixed with about 10% of the *dl* acid.

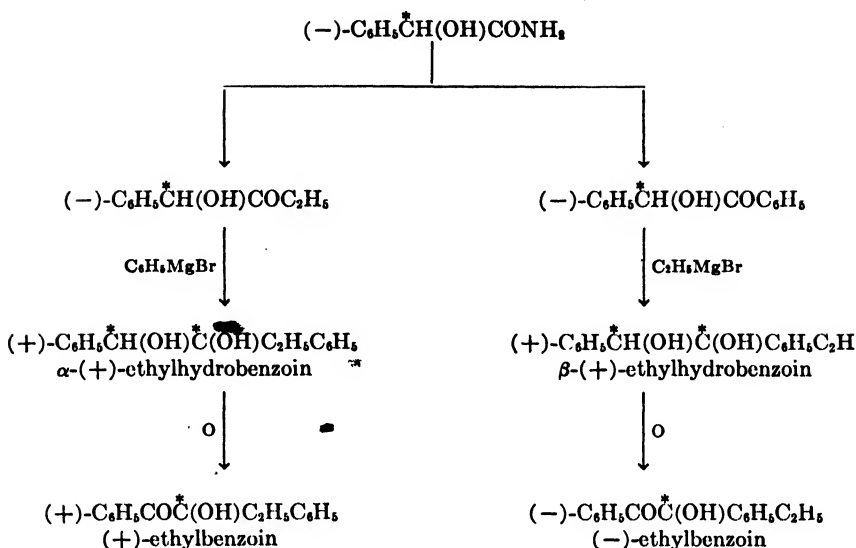
**(C3) Internal Asymmetric Synthesis (Noneliminative).** In asymmetric syntheses of the eliminative type the intermediate directing agency is completely removed from the final product after fixation of the asymmetry induced by it—for example, the (–)-menthyl esters are fully saponified, and the (–)-menthol removed by steam distillation. The present category, however, is of a different type. Several recent experiments have revealed and “isolated” the optical activity due to the fixation of intra-

molecularly induced asymmetry, not by eliminating the intermediate directing group as such, but by destroying its dissymmetry without actually removing it from the new molecule. It is conceivable, for example, that some process of selective racemization might inactivate the original directing system without affecting the newly generated system, though no example of this appears to have been reported. However, Kenyon and Partridge (58) have described the following series of changes:



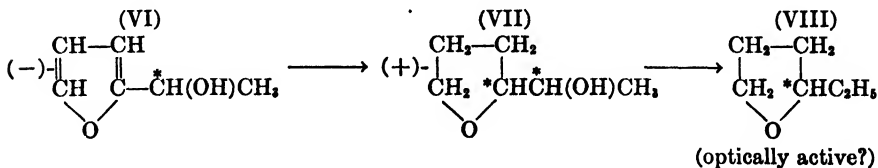
Bromination of I yielded diastereoisomerides II and IV, which were separated by fractionation and then oxidized individually. The antipodal substances, III and V, respectively, were isolated, in which the induced asymmetry of the original olefinic group has been fixed by bromination, but the original dissymmetric secondary alcoholic grouping has been oxidized to a symmetrical carbonyl group. It remains a part of the final molecule, but with its classical dissymmetry destroyed, though it probably still contributes to the total optical activity of the product through asymmetric induction.

Similarly, a series of changes (p. 88) have been described by Roger(124), who discussed his results in the light of Marckwald's definition of asymmetric synthesis, and proposed to call the reaction a "unilateral internal asymmetric synthesis." The directing group appears to have a completely unilateral inducing effect on the immediately adjacent carbonyl group, the final ethylbenzoin being optically pure. On the other hand, if the inducing effect had to be transmitted through an intervening chain of one or more carbon atoms, it is possible that it would be only partially unilateral, and that an optically impure product might result. To provide for the general as well as for the limiting case, the word "unilateral" might with some advantage be omitted from Roger's nomenclature. The effect



of inverting the order of introduction of the phenyl and ethyl groups has been discussed by Partridge (117), who pointed out the essential similarity between Roger's experiment and the synthesis by McKenzie and Ritchie of (+)- and (-)-methylanisylglycolic acids (90).

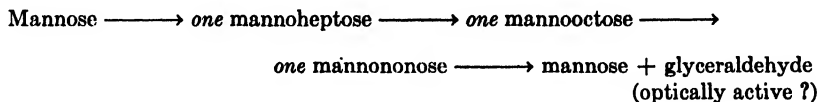
Duveen and Kenyon (32) attempted to carry out the following ingenious series of reactions which, had they been successful, would have provided another example of internal asymmetric synthesis:



The reduction of VI to VII was successfully achieved, thereby generating a new dissymmetric center; but various attempts to replace the hydroxyl group were without conclusive result. It is possible that an oxidation of VII might lead to the formation of an optically active methyl-2-tetrahydrofuryl ketone.

It is clear, therefore, that the earlier work of McKenzie and Wren (96) on the preparation from (-)-benzoin of optically pure  $\beta$ -(+)-methylhydrobenzoin,  $\text{C}_6\text{H}_5\text{CH}(\text{OH})\text{C}(\text{OH})\text{CH}_2\text{C}_6\text{H}_5$ , can be regarded as the first

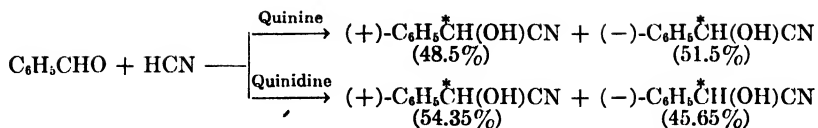
stage of such an internal asymmetric synthesis. The formation of only one of the theoretically possible diastereoisomeric mannoheptoses from mannose by the cyanohydrin synthesis can also be regarded in this way. It will be recalled that Fischer suggested that repetitions of this latter reaction, followed by a suitable fission of the original mannose molecule from the asymmetrically produced extension of the chain, might result in the asymmetric synthesis of an optically active glyceraldehyde, though this reaction has never been experimentally realized:



It has been suggested previously that the inducing effect of the directing group may become less unilateral when it is transmitted to the carbonyl group along a chain of intervening carbon atoms. This is reminiscent of recent views on the electronic mechanism of certain organic reactions, in which the polar influence of a distant substituent is also damped-out by transmission along a chain; and it receives some support from observations recorded by Tiffeneau and co-workers. Like McKenzie and Wren (96) and Roger (124), they noted the completely unilateral attack of a Grignard reagent upon a carbonyl group immediately adjacent to a dissymmetric carbon atom. When, however, as in (+)-phenyl  $\alpha$ -campholenyl ketone, (+)- $\text{C}_8\text{H}_{13}\text{CH}_2\text{COPh}$ , there is an intervening methylene group, Tiffeneau, Lévy, and Ditz (139a) conclude that the action of an appropriate Grignard reagent yields both the possible diastereoisomerides, though in unequal amount. As pointed out by Tiffeneau, and emphasized by the present writer (123), the experimental evidence for the formation of both diastereoisomerides is at one crucial point susceptible of two different steric interpretations, though it seems not unlikely that the published conclusion is the correct one.

**(C4) Catalytic Asymmetric Synthesis.** This reaction category is of fundamental interest to the biochemist, and as an approach to the question of enzymic catalysis we may first consider a few illuminating examples where catalysts of known constitution have been employed.

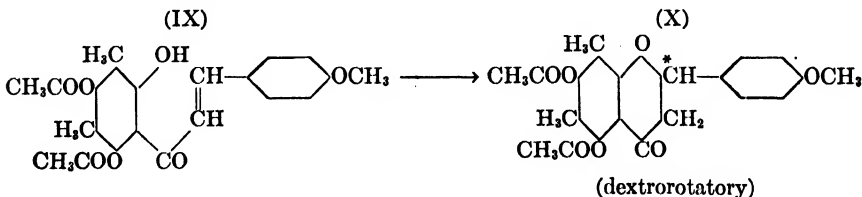
Bredig and Fiske (14) found that the formation of mandelonitrile from benzaldehyde and hydrogen cyanide, which is accelerated by basic catalysts, proceeded asymmetrically in the presence of quinine or quinidine, in either chloroform or toluene solution:



Similar results were obtained by Bredig and Minaeff (17) with five other aldehydes of very varying type. In all cases, only a small proportion of alkaloid was required, and the action of the latter therefore appeared to be truly catalytic. A colloidal adsorption complex of the reactants with the alkaloid is very probably the effective phase in this catalytic asymmetric synthesis, for, as Smith (134) has pointed out in a discussion of this work, Traube and Onodera (140) found that alkaloids of high molecular weight form colloidal solutions even in water, and hence probably also in the solvents used by Bredig and Fiske.

It seems not unlikely that a similar catalytic role is played by an alkaloid in the asymmetric synthesis of  $\beta$ -phenyl-*n*-butyric acid recorded by Lipkin and Stewart (76), by a reduction of the hydrocinchonine salt of  $\beta$ -methyleinnamic acid, and also in the optically selective bromination of cinchotine cinnamate and glucosamine cinnamate noted by Erlenmeyer (38). A more complicated example recorded by Stewart and Lipkin (136) may perhaps be an extension of the same principle; here,  $\beta$ -methyleinnamic acid was selectively reduced by glucose in the presence of Raney nickel.

Fujise and Sasaki (42) have recorded a peculiar result which appears to fall in this category. Ring closure of IX in the presence of (+)-camphorsulfonic acid (which presumably acts as an asymmetric catalyst) led to the optically selective formation of X, in which a dissymmetric carbon atom has been generated:

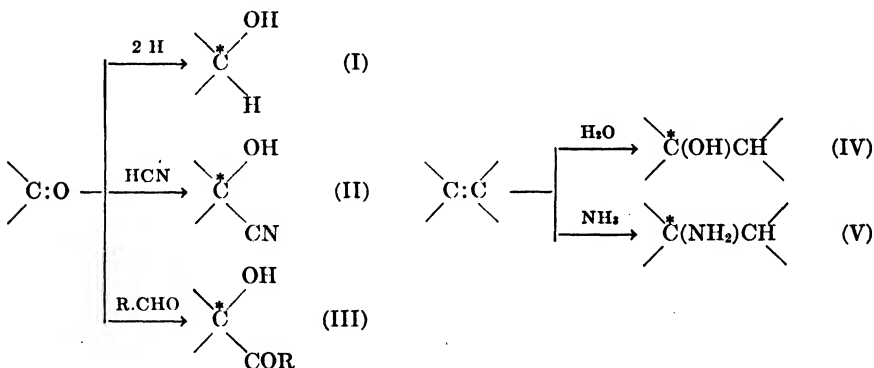


An ingenious application of these catalytic reactions has been made by Bredig and Gerstner (15) and Bredig, Gerstner, and Lang (16). A base such as diethylamine catalyzes the addition of hydrogen cyanide to benzaldehyde, but has, of course, no asymmetric effect. However, on introduc-

ing the diethylamino group into cellulose fibers, which are optically active, it was found that the diethylaminocellulose brought about a catalytic asymmetric synthesis of (–)-mandelonitrile in the same way as quinine. Here we have a colloidal substrate with specific active groupings attached, and this perhaps affords a molecular model for comparison with the catalytic mechanism of enzymic catalysts of unknown constitution.

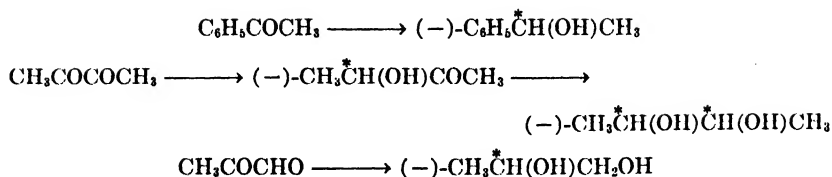
A wide range of such enzymic reactions may be effected either in living tissues or in the laboratory by the action of yeast, molds, bacteria, or cell-free enzymes. The enzymes are presumably optically active; and, though little is known of the mechanism of their reaction as catalysts, they most probably act as colloids by a surface reaction with a substrate of appropriate configurational "fit." How far one is justified in regarding such reactions as intermolecular, in contrast to the intramolecular effect in eliminative asymmetric synthesis (C2), is a moot point; it may well prove that eliminative and catalytic types of asymmetric synthesis (C2, C4) should be regarded merely as the ideal limiting cases of a series of continuously graded phenomena, all complying with Marckwald's definition.

However this may be, all the biochemical asymmetric syntheses considered here depend upon conversion of unsaturated groupings such as C:O and C:C into centers of fixed dissymmetry by a selectively catalyzed addition reaction of one of the following types:

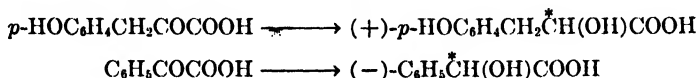


(a) *Asymmetric Phytochemical Reductions.* Neuberg and his school (109) are particularly associated with (I), which has come to be known as *asymmetric phytochemical reduction*. Many living cells contain reducing enzymes (reductases) by which catalytic asymmetric syntheses such as the following have been achieved:



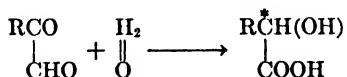


A technic much favored by Neuberg and others involves the addition of the carbonyl compound to a solution of sucrose undergoing yeast fermentation, but other methods are applicable. Thus, the following reactions have been carried out by the action of the mold *Oidium lactis* (127), and liver extract or yeast (62), respectively:



In general, these reductions are only partially, not completely, optically selective, though the formation of practically pure (+)- $\beta$ -hydroxy-*n*-butyric acid by the action of fermenting yeast on the potassium salt of ethyl acetoacetate has been claimed by Friedmann (41).

(b) *Ketoaldehyde Mutase Dismutations*. A further variation on reaction I (page 91) is the simultaneous reduction and oxidation of keto aldehydes, such as methylglyoxal, under the influence of the enzyme *ketoaldehyde mutase*. This very widely distributed enzyme, first recorded by Neuberg (108) and Dakin and Dudley (28), brings about the dismutation of the labile hydrates of such keto aldehydes by a species of intramolecular Cannizzaro reaction:



The production of the acid  $\text{R}\overset{*}{\text{C}}\text{H}(\text{OH})\text{COOH}$  normally occurs with high efficiency and almost complete optical selectivity. Thus, with phenylglyoxal, the action of *Acetobacter ascendens* gives pure (-)-mandelic acid in 95% yield, while a dry preparation of a lactic bacillus gives almost pure (+)-mandelic acid in 97% yield. Other equally striking examples have been recorded.

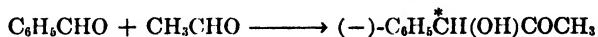
(c) *Oxynitrilase Syntheses*. Reactions of type II (page 91) have been studied, in particular, by Rosenthaler (126); they bear a striking parallel to the previously discussed results of Bredig and Fiske. Rosen-

thaler found that the enzyme *oxynitrilase*, present in almond emulsin, caused the optically selective addition of hydrogen cyanide to a great variety of aldehydes, the resulting cyanohydrin being in many cases hydrolyzed to an optically active hydroxy acid. Thus, benzaldehyde yielded impure (–)-mandelic acid. Krieble and Wieland (65) later worked out the conditions under which the intermediate (+)-mandelonitrile could be isolated in a fairly high degree of optical purity.

It has, further, been found that oxynitrilases of opposite stereospecificity exist in nature. Thus, the leaves of *Taraktogenos Blumei* Hssk. contain an oxynitrilase which so directs the addition of hydrogen cyanide to benzaldehyde that (–)-mandelonitrile is produced, yielding (+)-mandelic acid on hydrolysis.

Albers and Hamann (4) have studied the kinetics of the reaction, and conclude that enzymic and nonenzymic formation of cyanohydrin proceed concurrently, the oxynitrilase accelerating the rate of the optically selective process. The various views expressed on the mechanism of this reaction have been summarized by McKenzie (83).

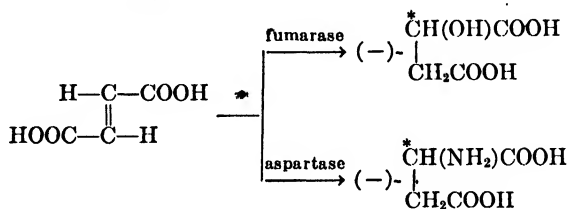
(d) *Carboligase Syntheses*. It will have been noticed that reactions of type II bring about the formation of a new carbon-to-carbon linkage. An even more striking example of this is provided by the reactions of type III, brought about by an enzyme said to be present in yeast to which Neuberg and Hirsch (110) assigned the appropriate name *carboligase*. If, for example, benzaldehyde is slowly added to an aqueous solution of sucrose undergoing fermentation by a variety of top yeast, a levorotatory ketol is produced as follows:



A possible mechanism is that the pyruvic acid produced during alcoholic fermentation under the influence of a carboxylase yields acetaldehyde in a nascent or specially reactive form, and that the latter then condenses with the benzaldehyde under the asymmetric catalytic effect of carboligase. There is much that still requires elucidation in this reaction, but it should at least be noted that this peculiar new aldol-type reaction cannot be effected by nonenzymic means.

The precise constitution of Neuberg's ketol gave rise to much discussion, and the elucidation of the problem has been well summarized by McKenzie (83). The reaction is not, of course, limited to benzaldehyde; other aromatic aldehydes such as *o*-chlorobenzaldehyde (111) and *o*- and *p*-tolualdehydes (8) give a similar result.

(e) *Other Enzymic Syntheses.* Reactions of types IV and V are less common. Type IV is exemplified by Dakin's observations that ammonium cinnamate, injected subcutaneously into dog tissues, yields a levorotatory  $\beta$ -phenyl- $\beta$ -hydroxypropionic acid as a product of metabolism (26), and that fumaric acid is converted by a muscle enzyme (fumarase) into (–)-malic acid (27). Similar catalytic asymmetric syntheses by fumarase are recorded by Jacobsohn (50), Challenger and Klein (20), and Clutterbuck (22). Type V is exemplified by the conversion of fumaric acid to (–)-aspartic acid, by means of the enzyme aspartase in beer yeast, as recorded by Sumiki (137):



#### D. OPTICALLY SELECTIVE INACTIVATION OF EXISTING CENTERS OF DISSYMMETRY

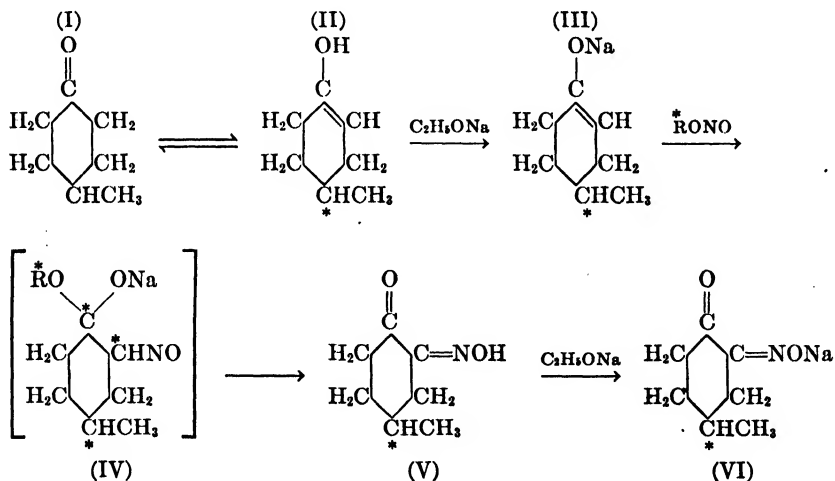
(D1) **Absolute Asymmetric Decomposition.** Several recorded experiments can be classified under this heading. In 1929, Kuhn and Braun (69) decomposed *dl*-ethyl- $\alpha$ -bromopropionate by circularly polarized light, and obtained an optically active product. The maximum observed rotation was only  $\pm 0.05^\circ$ , and the nature of the complex reaction was not elucidated. However, later, Kuhn and Knopf (70) obtained a more pronounced effect with *dl*- $\alpha$ -azidopropiondimethylamide. The course of the photodecomposition was here more fully elucidated; and irradiation with dextro- and levocircularly polarized light produced observed rotations as high as  $+0.78^\circ$  and  $-1.04^\circ$ , respectively—well beyond the limit of experimental error.

In a similar experiment, Mitchell (106,107) found that photochemical decomposition of humulene nitrosite by circularly polarized light gave an optically active product, of undetermined constitution, with a maximum observed rotation of  $\pm 0.30^\circ$ .

Tsuchida, Nakamura, and Kobayashi (142) have successfully extended the principle to the photochemical decomposition of racemic metallic co-ordination compounds. Irradiation of a solution of *dl*-[Co(C<sub>2</sub>O<sub>4</sub>)<sub>3</sub>]K<sub>3</sub> with dextrocircularly polarized light led to the development of a marked

levorotation, which passed through a maximum and then fell gradually to zero. This latter phenomenon was also observed by Mitchell (106) in his experiments, and is presumably due to the fact that we are dealing here with differential rates of reaction of antipodal molecules, originally present in equal amounts, so that *completion* of the reaction leads to the decomposition of an equal number of antipodal molecules. Only at an intermediate stage is the selective decomposition shown by optical activity.

**(D2) Eliminative Asymmetric Decomposition.** A result previously classed provisionally as a type of asymmetric synthesis may probably more properly be classed now under this new heading. Pezold and Shriner (118) found that the action of (+)-2-octyl nitrite on 4-methylcyclohexanone, in the presence of sodium ethoxide, gave a levorotatory sodium salt of 2-oximino-4-methylcyclohexanone, (+)-2-octyl alcohol being eliminated. Other analogous results have been recorded by Shriner and co-workers (48,131,139). Although Pezold and Shriner described this reaction as an asymmetric synthesis, the present author is of the opinion that it should be classed rather as an internal eliminative asymmetric decomposition, since the reaction very probably proceeds via the enolic form II of ketone I by the following mechanism:

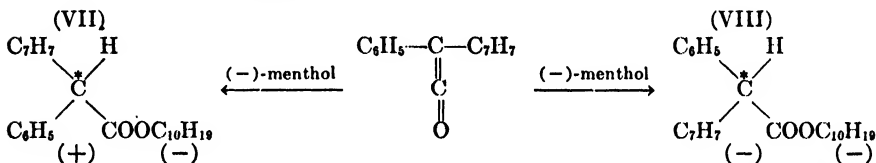


The case is admittedly complicated, for collision of the racemic sodium enolate (III) with the unsymmetrical addendum (\*RONO) generates two new dissymmetric carbon atoms in the presumed intermediate (IV). It seems probable that the addition proceeds to completion (unlike cases

A5 and A6), so that IV should exist as a transient mixture of two diastereoisomerides *in equal amounts*; but the next stage (elimination of  $\text{RONa}^*$ ) should be an asymmetric one, the oxime (V) and its salt (VI) being produced by an optically selective decomposition.

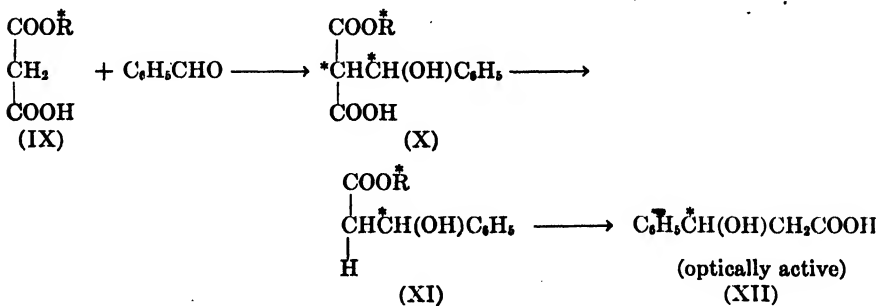
The result of Pezold and Shriner may instructively be compared with the work of Marckwald and McKenzie on the differential rates of saponification of the  $(-)$ -menthyl mandelates, and it would be interesting to determine whether the production of optical activity depends here also upon incomplete asymmetric decomposition of the diastereoisomerides of IV. A positive result ~~here~~ would throw further light on the precise reaction mechanism.

A variation of this idea, postulating the symmetrical addition of an optically active addendum ~~to~~ a symmetrical unsaturated molecule, has already been advanced in another connection. Weiss (146) claimed that the addition of  $(-)$ -menthol to phenyl-*p*-tolylketene yielded the ester (VII) free from its diastereoisomeride (VIII):



The present author criticized this as unlikely, on classical grounds (123); and this view has been confirmed by McKenzie and Christie (84), who proved the nonselective formation of *both* diastereoisomeric esters in a repetition of Weiss' experiment, and in two analogous cases.

An experiment which is difficult to classify, but which probably belongs to this category, is the synthesis of optically active  $\beta$ -phenyl- $\beta$ -hydroxypropionic acid XII reported by Abbott, Christie, and McKenzie (1):

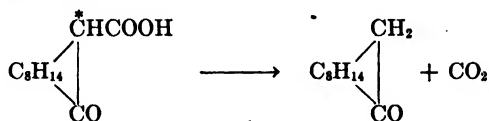


\*  
(R = (-)-menthyl or (-)-bornyl). The condensation of IX with benzaldehyde again involves the collision of an optically active addendum with a symmetrical, unsaturated molecule, so that X (in which two new dissymmetric carbon atoms  $\overset{*}{C}$  have been generated) most probably consists of a mixture of two diastereoisomerides in equal amounts. If this assumption is correct, the optical activity of the final product (XII) owes its existence to the optically selective inactivation of the dissymmetric carbon atom from which carbon dioxide is detached in the conversion of X to XI. Substance XI would then consist of two diastereoisomerides in unequal amounts, yielding optically active XII on saponification. It is, therefore, tentatively proposed to classify this reaction not as an asymmetric synthesis, but as an eliminative asymmetric decomposition.

(D3) **Noneliminative Asymmetric Decomposition.** No example of this type of reaction appears to have been recorded as such; but, if the reasoning in the previous paragraph is correct, the conversion of X to XII could be considered a rather complicated first instance.

(D4) **Catalytic Asymmetric Decomposition.** Here, as in the previously considered case of catalytic asymmetric synthesis, discussion of reactions *in vitro* with catalysts of known constitution will precede that of reactions *in vivo* with enzymic catalysts.

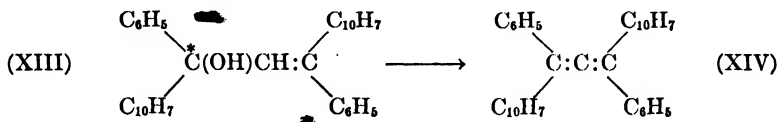
Bredig and Fajans (13) and Fajans (39) investigated the catalytic fission of *dl*-camphorcarboxylic acid into camphor and carbon dioxide by means of basic catalysts:



With optically active bases such as the alkaloids, the antipodal forms of the *dl* acid decomposed at unequal rates, and it was possible under appropriate conditions to isolate an optically active camphor. (This stereospecific "Abbau" is not an asymmetric *synthesis*, since a dissymmetric carbon atom  $\overset{*}{C}$  in the original acid molecule is destroyed without generation of a new one in the product.) Critical analysis of the results showed the reaction to be a true catalysis—not a salt, but a transient intermediate complex of acid and colloidal catalyst was formed, with subsequent regeneration of unchanged alkaloid.

A different type of catalysis was achieved by Wuyts (148), who found

that *dl*-phenylmethylcarbinol undergoes catalytic dehydration at 100°C. in the presence of only 1% (+)-camphorsulfonic acid, the two antipodes decomposing at different rates. At any stage short of complete dehydration, therefore, an optically active carbinol is present. This reaction has been given a very interesting application by Maitland and Mills (97) in preparing the (+)-allene (XIV) by catalytic dehydration of the racemic olefinic carbinol (XIII), by heating in benzene solution with (+)-camphor-sulfonic acid as a catalyst; with the (−) acid as a catalyst, the (−)-allene is obtained:



This result is very similar to that achieved by Bredig, each involving optically selective catalytic decomposition of the two antipodal molecules. It may be noted that Bousset (11) failed to achieve an optically selective dehydration of 4-methylcyclohexanol by the use of camphoric anhydride.

An entirely different case is provided by the optically selective oxidation of racemates in the presence of optically active co-ordination compounds as catalysts. Shibata and Tsuchida (130), for example, oxidized *dl*-3,4-dihydroxyphenylalanine in the presence of (−)-[Co(en<sub>2</sub>, NH<sub>3</sub>, Cl)]Br<sub>2</sub> and found that the (−) antipode of the amino acid was preferentially destroyed.

Turning to the use of enzymic catalysts of unknown constitution, we find, as possibly the earliest recorded example, Pasteur's biochemical "resolution" of ammonium *dl*-tartrate by the mold *Penicillium glaucum*; and an analogous observation that the oxidases from various molds oxidize (+)-tartaric acid more rapidly than its antipode was recorded by Herzog and Meier (46). The oxidative deamination of α-amino acids investigated by Krebs (63) and Kisch (60) shows a similar effect.

One feature of the above results of Bredig and Fajans (13) merits additional comment. It was observed by Bredig and Balcom (12) that (+)- and (−)-camphorcarboxylic acids decompose with equal velocity into camphor and carbon dioxide when dissolved in either (+)- or (−)-limonene—a marked contrast to the result when the decomposition is catalyzed by an alkaloid. It appears that an optically active solvent does not affect the decomposition of a racemic solute in an optically selective manner unless it is a true catalyst for the decomposition in question (or contains such a

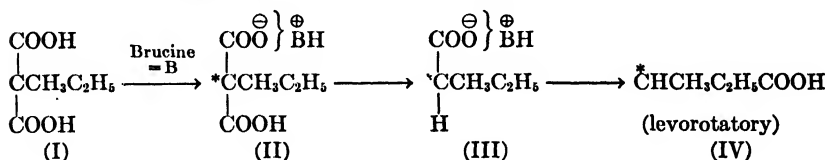
catalyst in solution). This principle may explain the almost complete failure of many experiments—for example, those designed to detect a difference in the solubility of antipodal molecules in an optically active solvent (150). Further confirmation of the basic idea has recently been provided by Tarbell and Paulson (138), who examined and refuted the claim of Betti and Lucchi (10) that they prepared optically active phenylmethylcarbinol by the action of methyl magnesium iodide on benzaldehyde in an optically active solvent such as dimethylbornylamine.

### E. SOME UNCLASSIFIED EXPERIMENTS

It remains now to mention one or two experiments which are difficult to assign with certainty to any of the above categories, largely because certain experimental data are still lacking.

(a) *Marckwald's "Asymmetric Synthesis" of (-)-Valeric Acid.* It is most curious that the first experiment to be claimed as a definite asymmetric synthesis does not appear to be strictly assignable to any of the above classes—namely, Marckwald's synthesis of optically active methylethylacetic acid (valeric acid).

Marckwald (98), it will be recalled, converted methylethylmalonic acid (I) into its acid brucine salt (II), thereby generating a new dissymmetric carbon atom C\*. He then heated this salt to eliminate carbon dioxide, removed the directing agent (the alkaloid) from III, and obtained a product, IV, consisting of about 55% (-) acid and 45% (+) acid:



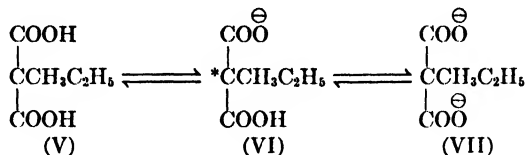
There has been much discussion of this peculiar experiment, which may be summed up as follows:

(1) Cohen and Patterson (23) contended that the original experimental conditions might have led to analytical separation of the diastereoisomeric forms of II, the final result being due therefore to partial resolution, not to asymmetric synthesis. Erlenmeyer and Landsberger (37), however, repeated the experiment under modified conditions, expelling the solvent entirely from an alcoholic solution of brucine (1 M) and methylethylacetic acid (1 M); here, though no resolution was possible, Marckwald's result was again observed.

(2) Cohen and Patterson (23) also contended that solutions of acid I already contained dissymmetric ion VI before combination with brucine, and that no entirely new



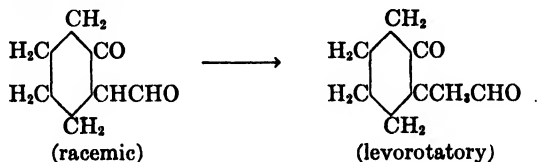
dissymmetric center was generated in the synthesis. Marckwald (99) replied that the same result could be obtained using nonionizing solvents, though it is doubtful whether a quaternary ammonium salt such as II can be regarded as unionized in chloroform:



(3) Eisenlohr and Meier (34), after a thorough reinvestigation of the whole problem, concluded that in solution, or in the noncrystalline viscous state, the two diastereoisomerides of II are present in equal amount, and decompose with equal velocity on heating. They attributed Marckwald's result to a shifting of the equilibrium between these diastereoisomerides when crystallization begins, in accordance with their different solubilities. Hence, even though both decompose at the same rate, their solubility relationships have already dictated that a final excess of one antipode shall be formed.

If Eisenlohr and Meier are correct, the result must be classed not as an asymmetric synthesis but as a second-order asymmetric transformation. The diastereoisomerides of II have no real existence in solution; but as soon as crystallization sets in, the less soluble would be expected to separate out preferentially. There is, however, a complicating factor here which still makes it uncertain how this synthesis should be classified. Diastereoisomerides should differ significantly in all their physical properties, and it is difficult to accept Eisenlohr and Meier's assertion that the diastereoisomeric forms of II eliminate carbon dioxide at the same rate. It seems likely that the final optical activity is the resultant of two factors—a more or less one-sided asymmetric transformation, followed by optically selective breakdown of diastereoisomerides.

(b) *An Unconfirmed "Absolute" Asymmetric Synthesis.* A very astonishing result was recorded in 1943 by Paranjape, Phalnikar, Bhide, and Nargund (116). They claimed that by the action of sodium and methyl iodide on 2-formylcyclohexanone an optically active 2-methyl-2-formylcyclohexanone was obtained:



Nothing further appears to have been published by these workers in support of this surprising claim, though an attempt by O'Gorman (114) to

repeat the experiment led, not unexpectedly, to a completely negative result. Should the original claim be confirmed, it appears to be an inexplicable result unless we are prepared to accept the alibi of a completely chance synthesis; further comment must be withheld pending a fuller investigation of the report.

## V. Maintenance of Optical Purity in Living Organisms

Since the separation of a racemate into its antipodal forms requires an expenditure of work, and since the recombination of the latter to form a racemate liberates energy correspondingly, it is apparent that a racemic system is thermodynamically more stable than its separated antipodes. It is, nevertheless, a commonplace that natural tissues exhibit a high degree of optical specificity. We have therefore to consider how it is that the metabolism of a living organism contrives to favor optically pure protoplasmic systems rather than the more thermodynamically stable racemic systems which, possessing a lower amount of free energy, are found in inorganic nature. There must obviously be two competing mechanisms at work, one tending toward racemization, and one tending to evade or correct it.

### A. CHARACTERISTICS OF ASYMMETRIC CATALYSIS

In 1936 Kuhn (68) published a very instructive analysis of the kinetics of catalytic asymmetric syntheses and decompositions, which has a direct bearing on the above-mentioned racemization tendency. He confirmed, in the first place, that the kinetics of Rosenthaler's oxynitrilase synthesis showed all the criteria of true catalysis. The equilibrium constant, for example, was not dependent upon the catalyst; addition of catalyst to a reaction system in which symmetrical nonenzymic nitrile formation was already in progress did not affect the product formed up to that point, but rendered subsequent nitrile formation optically selective; and finally, as observed by Rosenthaler and by Nordefeldt (113), the optical activity produced was only temporary, rising from zero to a maximum, and then returning asymptotically to zero as the reaction approached completion. McKenzie has, it is true, pointed out (83) that this loss of optical activity might well be due to the very ready racemization of mandelonitrile, as recorded by Smith (132); however, irrespective of any such racemization, Kuhn showed theoretically that in a true asymmetric catalysis one antipode will be formed initially at a greater rate than the other, but that the excess will gradually decrease as the reaction progresses until a racemate results.

Furthermore, he showed that these considerations apply to catalytic asymmetric syntheses and decompositions alike, though in the former the stability of the optically active state (measured by the ratio of the time required for racemate formation to the time required for attainment of maximum optical activity) will be considerably greater than in the latter.

#### B. EVASION AND CORRECTION OF RACEMIZATION TENDENCIES

To secure constancy of optical purity, and to evade for as long as possible the ultimately inevitable racemization (which Kuhn tentatively envisages as part, at least, of the process of biological aging), nature appears to employ several selective mechanisms.

In the first place, in an enzymic catalysis, the ratio of the reaction velocities leading to the two antipodal forms of the new dissymmetric molecule is very high, often of the order of 1000:1 or more. In nonenzymic asymmetric catalysis, on the other hand—for example, in the work of Bredig and Fiske—the ratio is very much less, generally of the order of only 2:1. Correspondingly the equilibrium constant is greater, and the optical stability of the product higher.

Nevertheless, if the final racemic end point is to be avoided for very long, this initial evasive mechanism requires supplementation by a corrective mechanism. Krebs (63) and Kisch (60) have confirmed that such a corrective mechanism exists whereby the initial preponderance of one configurational series could be maintained by selective destruction of the unwanted series. They found that oxidative deamination of  $\alpha$ -amino acids by a deaminase present in animal liver and kidney tissues is markedly optically selective, the "unnatural" *d*-series being deaminated more rapidly than the "natural" *l*-series. This process will continuously correct and regulate the inevitable tendency for at least some of the unwanted antipode to appear in a true catalytic asymmetric synthesis.

Another such corrective process has been discussed by Langenbeck and Triem (71). They showed theoretically and confirmed experimentally that, if two optically impure substances, A and B, undergo a process of combination which is interrupted before completion, the four possible diastereoisomerides are not found in equivalent amounts. If, to quote an example,  $[(-)-A] > [(+)-A]$  and  $[(-)-B] > [(+)-B]$  in the original mixture, then on interruption of the reaction before completion we will have:

$$\frac{[(-)-A (-)-B]}{[(+)-A (+)-B]} > \frac{[(-)-A]}{[(+)-A]} \quad \text{and} \quad \frac{[(-)-A (-)-B]}{[(+)-A (+)-B]} > \frac{[(-)-B]}{[(+)-B]}$$

In other words, the optical purity of the transformed material is increased, though that of the residual untransformed material is correspondingly decreased.

Developing this idea, we arrive at the conclusion that the succession of interrupted syntheses and degradations occurring continuously in biological systems may be added to the list of mechanisms which help such systems to evade or correct the racemization process. As Gause (43) puts it, "the incessant reconstruction of living matter should. . . , perhaps, be considered as an indispensable condition for the maintenance of the optical purity of stereo-autonomic substances."

In passing, it is worth noting that Astbury has recently presented a wholly admirable survey of the way in which, according to modern views, the optically pure and highly stereospecific long-chain protein molecules may act as a template to direct the subsequent biosynthesis of other macromolecular structures, such as the polysaccharides and nucleic acids. It is perhaps permissible to quote one highly suggestive sentence apart from its closely reasoned context (6):

"It may be only a numerical coincidence, yet it is a fact that the period of a glucose residue along the cellulose chain is practically identical with the length of an  $\alpha$ -type fold in a polypeptide chain; and the possibility therefore occurs to one that the glucose molecules are first strung out by temporary combination with successive corresponding side-chains in a long  $\alpha$ -template, then condensed at the glucosidic linkages, and finally liberated from the template as a continuous polysaccharide."

### C. BIOLOGICAL ADVANTAGES OF OPTICAL PURITY

We may here consider briefly what advantages are obtained by living organisms through working with optically pure rather than racemic substances. Advantages there must be, if we are to explain on the basis of natural selection the evolution of such highly efficient and specialized evasive and corrective mechanisms as those just discussed.

Gause (43) points out that:

"... the evolution of living beings has consisted in a gradual increase in the number of fixed parameters in the internal milieu. . . . The elaboration of mechanisms in living matter to maintain optical purity evidently contributes to the fixity of [this] internal milieu. The spatial parameters which determine the asymmetry of a substance are fixed in primary constituents of protoplasm in such a way that optical purity is maintained."

Mills (102) has outlined persuasive though avowedly tentative arguments, based on a cautious application of the law of mass action to enzymic

processes, showing that this should lead to greater efficiency and rapidity of tissue growth than with racemic substances. Part of his argument is worth quoting verbatim:

"Let us consider the hydrolysis of dextro-sucrose by invertase, at not too great concentrations, so that the velocity of hydrolysis is approximately proportional to the concentrations both of substrate and of enzyme, and compare the initial rate of inversion with what it would be if we employed instead inactive materials at the same total concentrations—*dl*-sucrose and inactive invertase. From the known specificity of the action of invertase we may safely assume that ordinary invertase would activate dextro-sucrose only and that its mirror image would activate laevo-sucrose only. Then it is clear that in the first experiment every sucrose molecule that encountered invertase would be susceptible of activation by it, whereas in the second only half the encounters would be potentially effective and the reaction would proceed at only half the rate at which it takes place in the optically active mixture. . . If . . . we are justified in assuming that in living matter concentrations [beyond which the law of mass action cannot be applied] are not approached, and that diminutions in concentrations of a molecular species would be accompanied by an approximately proportional fall in the velocity of the reaction or reactions in which it was concerned, then the inactivation of living matter by the instantaneous replacement of half of each of its optically active components by their enantiomorphs would suddenly diminish the rates of all the stereospecific reactions proceeding in it to rates approximating more or less, in the case of reactions of bimolecular type, to one-half of their former magnitudes."

## VI. Physical vs. Statistical Origin of Optical Activity in Nature

As has already been shown, several quite different mechanisms may operate in optically selective biosynthesis; and, given the initial presence of an optically active substance, there is no difficulty in accounting for the perpetuation of asymmetry through a subsequent chain of biosyntheses. But we have still to face the thorny problem of the production of the initial directing agent in an optically active state, and we may conclude this survey by considering the relative merits of the two rival mechanisms which have been proposed to account for this.

The first of these—the intervention of an unsymmetrical physical directive agency such as circularly polarized light—has already been considered when dealing with absolute asymmetric syntheses and decompositions. Of recent years, however, attention has been widely directed to the statistical treatment of material systems and processes. Spiers (135), for example, has advanced arguments in support of the statistical or chance deviation mechanism; a statistical approach to the problem has also been advocated by Mills (102). It has much to commend it. The basic idea is that, in an aggregate of dissymmetric molecules, synthesized *in vitro* under

perfectly symmetrical conditions, the antipodal forms are in general so nearly equivalent in number that the medium is optically inactive when examined by any method at present available, but that the apparent numerical equality really represents a statistical mean around which fluctuations will occur in different experiments in accordance with the laws of probability. Mills quotes, as a concrete example, the synthesis of ten million dissymmetric molecules under symmetrical reaction conditions, and calculates that:

"...there is an even chance that the product will contain an excess of more than 0.021 per cent of one enantiomorph or the other. It is practically impossible for the product to be absolutely optically inactive."

If this is so, and if Mills's arguments supporting the greater efficiency of reaction between optically pure systems as contrasted with racemic systems are justified, then this admittedly very slight statistical departure from strict numerical equality

"...would increase with growth continually, according to a compound interest law until, eventually, the system originally in slight defect was completely swamped by its enantiomorph. From this point of view the optical activity of living matter is an inevitable consequence of its property of growth."

The two explanations—asymmetry due to a physical directing agency, and asymmetry due to a statistical effect—have each of them merits and defects, and no clear-cut decision between them is at present possible. Both, in fact, may operate. The physical explanation has the merit that it relates the almost completely unilateral stereochemistry of natural products—for example, the apparent nonexistence in nature of (–)-glucose, (+)-malic acid, and (+)-menthol—to a directing agency of apparently constant asymmetric sense; for dextrorotatory polarized light predominates at the earth's surface in reflected sunlight (19). The statistical explanation, on the other hand, has the weakness that, unless we assume that all living matter can be referred back to one single original microscopic particle with a dextro or a levo asymmetry, this unilateral stereochemistry is difficult to explain. If life originated in several of these primordial particles, arising at different places and times, there appears to be every chance that some would have imparted a dextro bias and some a levo bias, in more or less haphazard fashion, to subsequent biosyntheses originating from them.

One must, however, admit the justice of Mills's skepticism of an explanation based wholly on the action of circularly polarized light. Only

a minute fraction of the total illumination received by an organism under natural conditions can be circularly polarized; yet, even under the favorable and "concentrated" conditions of the laboratory, very great difficulty has been experienced in demonstrating a measurable activation by means of circularly polarized light. Furthermore, the efficiency of photochemical reactions in general is dependent upon the wave length of the irradiation. In absolute asymmetric photochemical reactions of the type demonstrated by Kuhn, there is a well-marked optimum wave length, and this factor still further reduces the potential effectiveness of circularly polarized white light as an activating agency.

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# SOME APPLICATIONS OF RADIOACTIVE INDICATORS IN TURNOVER STUDIES

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**I. Introduction**

Radioactive indicators have found extended application in the study of the absorption, excretion, circulation, and distribution of the elements and in the study of their turnover (84). In this chapter, much of the work carried out by using radiophosphorus, radiosulfur, and radioiodine as tracers in turnover studies is discussed. Because of the importance of phosphorus in many different metabolic processes and also in view of the easy availability of radiophosphorus, its convenient half-life period, and the penetrating power of the radiation emitted, radiophosphorus has found the most extended application among the radioactive indicators. Among

other radioactive tracers, both radioiodine and radiosulfur have, however, also been used in numerous studies, although radiosulfur is more difficult to prepare than radiophosphorus and radioiodine, and the  $\beta$ -rays emitted by radiosulfur are very soft which makes the determination of this isotope a more exacting task. The recent, improved means of preparing radioactive isotopes may more or less wipe out the differences in the availability of the different radioactive isotopes, but not the differences in the absorbability of the radiation emitted by them; and radiosulfur can still be expected to remain a tracer less favored by the workers in the field of radioactive indicators.

## II. Turnover of Phosphorus-Containing Carbohydrates\*

**Rate of Turnover.** The lack of interchange of atoms present in organic binding (hydrogen atoms bound to oxygen or nitrogen being an exception) (23), such as that of carbon atoms in glycogen or phosphorus atoms in lecithin with other carbon or phosphorus atoms, respectively, was found to be of great significance for the application of isotopic indicators in biochemical research. Owing to the absence of such interchange, the presence of labeled carbon atoms in glycogen molecules, or of labeled phosphorus atoms in lecithin molecules, extracted from the organs is to be interpreted as indicating the synthesis of these molecules in the course of the experiment in which the labeled atoms were administered. We can thus distinguish between "old" and "new" molecules and determine the rates at which molecules of the different compounds are built up in, or carried to, the different organs. For example the question—if and at what rate molecules of creatinephosphoric acid are enzymically dephosphorylated and subsequently rephosphorylated in the resting muscle—can hardly be answered without making use of an isotopic indicator. The same consideration applies to a large extent to the solution of such problems as that of the formation of thyroxine in the organs of thyroidectomized animals and numerous other processes discussed in this chapter.

### A. RATE OF RENEWAL OF LABILE PHOSPHORUS COMPOUNDS OF MUSCLE TISSUE

In the determination of the rate of renewal of creatinephosphoric acid *i.e.*, the rate of rephosphorylation of creatine molecules in muscle tissue,

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\* Investigations on the turnover of radiophosphorus in bone tissue and the application of radiophosphorus to labeling of red corpuscles are discussed in the monograph of the author (84).

we compare the radioactivity of 1 mg. of creatinephosphoric phosphorus at the end of the experiment with the average activity of 1 mg. of inorganic intracellular phosphorus prevailing during the experiment. If this ratio is found to be 0.2, we can conclude that 20% of the creatinephosphoric acid molecules present were renewed in the course of the experiment. Quantitative determination of rate of renewal is not an easy problem. It is not the activity of inorganic *intracellular* phosphorus that we determine experimentally, but the activity of inorganic *tissue* phosphorus. We calculate the former quantity from the latter by making the following assumptions:

(1) Extracellular space (chloride space) of muscle has the same free phosphate concentration as plasma, and (2) rate of interchange between plasma phosphate and extracellular phosphate is a comparatively rapid process, that is, the specific activity of free extracellular phosphorus is identical with that of free (inorganic) plasma P. If the specific activity of the free plasma phosphorus is equal to 1000 and that of the free tissue phosphorus to 100 counter units, and, if 20% of the muscle tissue is composed of extracellular fluid, the free phosphorus content of the plasma is 3 mg. per cent and that of the muscle tissue is 15 mg. per cent; then 0.6 mg. of 15 mg. free phosphorus will be extracellular phosphorus; the extracellular phosphorus will have an activity of 600, and the free tissue phosphorus of 1500, counter units. The 14.4 mg. free cellular phosphorus will thus have an activity of 900 units; or the specific activity of the free cellular phosphorus (62.6) will amount to only 62% of the experimentally determined specific activity of free tissue phosphorus (86). In experiments of shorter duration, which are often of great interest, the difference between the specific activity of the free tissue phosphorus and the free cellular phosphorus may become very appreciable. In such experiments even the assumption of equal specific activities for the inorganic extracellular phosphorus and the inorganic plasma phosphorus will not be justified.

One way of studying rate of renewal of phosphate compounds in muscle is by removal of the extracellular phosphate by perfusing muscle with saline. Kalckar and his associates (102) compared, under these circumstances, the specific activity of phosphocreatine phosphorus and of adenylypyrophosphate phosphorus with that of the inorganic phosphorus, which is exclusively of intracellular origin.

That in perfused muscle the specific activity of inorganic phosphorus is much lower than in unperfused muscle is seen from Table I. We must expect that perfusion will not influence the specific activity of the phosphocreatine and 2,3-pyrophosphate phosphorus, since these compounds are located in the cells.

Only twenty minutes after an intravenous injection of labeled phosphate, the creatine phosphorus, both of the rabbit's and the frog's (at 20°C.) skeletal muscle, was found to have a specific activity corresponding

TABLE I  
SPECIFIC ACTIVITY OF THE PHOSPHORUS FRACTIONS ISOLATED FROM THE SKELETAL  
MUSCLE OF THE RABBIT (102)

Type of injection of $^{32}\text{P}$	Time after injection, min.	Inorganic P		Phospho-creatine P		2,3-Pyrophosphate P	
		Unperfused	Perfused	Unperfused	Perfused	Unperfused	Perfused
Intravenous	30	235	100	61	61	58	57
Intraperitoneal	180	160	100		100	94	94

to 60% of that of the inorganic phosphorus. The same results were obtained for the labile phosphorus of adenylypyrophosphate, conforming with those of early experiments (79), and indicating that the rate of renewal of the labile phosphorus compounds is a rapid process, in contrast to the rate of penetration of labeled phosphate into the muscle cells.

In liver and red corpuscles (see page 124), the rate of rejuvenation of the labile adenylypyrophosphate phosphorus is of about the same order of magnitude as that in resting muscles. Five minutes after intravenous injection of labeled phosphate, the specific activity of the pyrophosphate phosphorus amounts to 83% of that for the inorganic phosphorus, and a similar figure was found for pyrophosphate present in red corpuscles of the rabbit (87).

Flock and Bollman (52,53), using myosin adenosine triphosphatase (ATPase) as a tool for differentiating between the two labile groups of ATP, have found, in experiments lasting one hour, a higher  $^{32}\text{P}$  concentration in the terminal phosphate group than in the second phosphate group (Table II). After the lapse of one day, the specific activity of the two

TABLE II  
SPECIFIC ACTIVITY OF THE THREE PHOSPHORUS ATOMS  
OF ADENOSINE TRIPHOSPHATE (53)

Fraction	Specific activity
Total inorganic P	100
Terminal adenosine triphosphate P	29
Second adenosine triphosphate P	21.6
Third adenosine triphosphate P	0.9
Creatine P	24

labile phosphorus atoms of ATP (see also reference 154) and that of the creatine phosphorus was found to be but slightly lower than the activity of



the inorganic phosphorus. The low rate of renewal of the third ATP phosphorus atom had been previously observed by Korzybski and Parnas (110).

In the turnover of the labile phosphate of ATP which occurs in the working muscle, the uptake of  $^{32}\text{P}$  is increased only slightly above that of the resting muscle, even after 180 contractions per minute for one hour. The turnover rate of the labile phosphate compounds is already so high in resting muscles that it is difficult to discover any substantial increase in the turnover during or after muscular contraction. The rate of rejuvenation of adenylypyrophosphate phosphorus and of phosphocreatine amounts to 20–30  $\mu\text{g.}$  per minute per g. of muscle (102). The rate of penetration of  $^{32}\text{P}$  into the cells of working muscle is not substantially different from the corresponding rate in resting muscle.

The rate of renewal of creatine phosphate and ATP molecules in cardiac muscle slices was investigated by Furchgott and Shorr (63). A quantitative renewal of creatinephosphoric and terminal ATP phosphorus was found to take place within 30 minutes at  $37.5^\circ\text{C.}$  (22a).

The extent of renewal of creatine and of the labile phosphorus of ATP molecules was found, even at  $2^\circ$ , to be appreciable, amounting to about one-third the values found at  $37.5^\circ$ . The rate of renewal in these experiments is calculated from the ratio of the specific activities of creatine phosphorus or of labile ATP phosphorus, and that of inorganic phosphorus after a prolonged washing of the tissue slices to remove the inorganic extracellular phosphorus. Such a procedure may lead to decomposition of the less active phosphorus compounds with formation of inorganic phosphorus and thus to a decrease in the specific activity of the inorganic phosphorus present after washing is terminated. Should this be the case, it will result in too high values for the renewal figures.

As for the labile phosphate split off ATP, it was found that it contained a mixture of the terminal, highly active, and a second, less active fraction. On splitting off the terminal phosphate with an enzymic crayfish muscle suspension free of phosphate, Furchgott and Shorr obtained, after 30 minutes, a fraction having a specific activity of 100, while only one-half of the remaining labile phosphorus of ATP underwent renewal in the course of the experiment.

In the boundary between the extracellular and the intracellular phases, a more or less continuous drop in the concentration of the introduced labeled phosphate may take place. If a phosphorus compound is built up inside the phase boundary, it is very difficult to determine the specific

activity of the inorganic phosphorus that has been utilized in the synthetic process resulting in the compound. Sacks (150,152) found that, in experiments on fasted animals taking a few hours or less, the specific activity of the glucose-6-phosphate phosphorus was very much higher than those of the phosphocreatine, ATP, and fructose-6-phosphate phosphorus, as seen in Tables III and IV. See also the results obtained by Kalckar and his associates (102). This result suggests that in the synthesis of the

TABLE III

TIME COURSE OF UPTAKE OF  $^{32}\text{P}$  BY ACID-LABILE GROUPS OF ADENOSINE TRIPHOSPHATE, FRUCTOSE-6-PHOSPHATE, AND GLUCOSE-6-PHOSPHATE IN RESTING MUSCLES OF FASTED CATS (150)\*

2 hr. after $^{32}\text{P}$			4 hr. after $^{32}\text{P}$			24 hr. after $^{32}\text{P}$		
ATP	Fructose-6-phosphate	Glucose-6-phosphate	ATP	Fructose-6-phosphate	Glucose-6-phosphate	ATP	Fructose-6-phosphate	Glucose-6-phosphate
38	65	124	75	31	114	193	132	84
80	84	202	84	14	167	167	122	102
66	27	149	52	19	170	165	121	88
79	37	189	77	25	219	224	150	121
88	102	209	78	60	228			
			65	56	250			
			70	45	217			
70	63	175	72	36	195	187	131	99

\* Values are expressed in terms of counts per minute per milligram P, calculated to the basis of  $10^6$  counts injected per minute per kilogram body weight, as of the day of measurement. Average values are italicized.

TABLE IV

UPTAKE OF  $^{32}\text{P}$  BY FASTING MUSCLES OF FROGS (150)\*

ATP	Fructose-6-phosphate	Glucose-6-phosphate	ATP	Fructose-6-phosphate	Glucose-6-phosphate
2 hr. after $^{32}\text{P}$			24 hr. after $^{32}\text{P}$		
73	82	348	233	142	134
23	62	152	306	155	141
84	154	435	152	100	99
136	144	472	106	62	65
79	111	352	199	115	109

\* Values are expressed in terms of counts per minute per milligram P, calculated to the basis of  $10^6$  counts injected per minute per kilogram body weight, as of the day of measurement. Average values are italicized.

labeled glucose-6-phosphate molecules no intracellular free phosphate, but phosphorus of much higher activity, is utilized, and thus that the synthesis of this compound takes place outside the muscle cells, *i.e.*, inside the extracellular space or possibly inside the phase boundary. The high  $^{32}\text{P}$  content of glucose-6-phosphate is never found in fed animals (in the post-absorptive phase) but only in fasted animals. The incorporation of non-intracellular  $^{32}\text{P}$  in glucose-6-phosphate phosphorus is thus presumably involved in the process of entry of glucose into cells of the fasting animal.

The rate of renewal of hexose monophosphate was found to be considerably lower than that of pyrophosphate or creatine phosphate (79,149).

\* TABLE V

EFFECT OF STIMULATION, RECOVERY, AND GLUCOSE ADMINISTRATION ON  $^{32}\text{P}$  TURNOVER IN MUSCLES OF CATS IN POSTABSORPTIVE STATE (151)\*

Phospho- creatine	ATP	Fructose- 6- phosphate	Glucose- 6-phos- phate	Phospho- creatine	ATP	Fructose- 6- phosphate	Glucose- 6-phos- phate
Resting				Stimulated			
119	98	61	77	99	102	41	54
130	132	76	59	138	125	69	77
170	143	...	47	204	160	105	44
157	150	133	73	104	115	...	76
<i>144</i>	<i>131</i>	<i>90</i>	<i>64</i>	<i>136</i>	<i>126</i>	<i>72</i>	<i>63</i>
Resting				Stimulation and recovery			
146	145	73	58	308	282	100	72
137	140	64	45	207	177	126	73
208	172	110	67	263	234	168	83
124	115	75	55	157	174	128	65
<i>154</i>	<i>143</i>	<i>81</i>	<i>56</i>	<i>234</i>	<i>217</i>	<i>131</i>	<i>73</i>
After glucose							
67	58	55	38	89	85	75	74
131	76	51	37	147	118	41	59
89	87	67	70	90	101	71	75
80	87	67	60	124	108	78	70
46	51	32	47				
39	56	17	30				
105	99	...	38				
75	92	50	94				
<i>79</i>	<i>76</i>	<i>48</i>	<i>53</i>	<i>113</i>	<i>103</i>	<i>66</i>	<i>70</i>

\* Values are expressed as counts per minute per milligram P, calculated to the basis of one million counts per minute injected per kilogram body weight. Average values are italicized.

Analysis of frog muscle at low temperature shows that the hexose monophosphate in skeletal muscle attained only a very low concentration of  $^{32}\text{P}$ . It appears as if some enzymes converting glycogen and phosphate to hexose monophosphate were inactive in frog muscle at low temperature (102).

Sacks (151,153) also studied the effects of prolonged stimulation, recovery, and glucose administration on the uptake of  $^{32}\text{P}$  by the phosphorus compounds of muscle of cats in fasting and postabsorptive state. Prolonged contraction was found to be without effect on either the uptake of  $^{32}\text{P}$  by any of the acid-soluble organic phosphorus compounds of muscle (see Table V), or its distribution among them. In recovery from prolonged activity all the organic phosphorus compounds investigated were found to show a higher  $^{32}\text{P}$  content, as seen in Table V.

Administration of glucose reduces the turnover rate in postabsorptive state of phosphocreatine, adenosine triphosphate, and fructose-6-phosphate, but not that of glucose-6-phosphate. In fasting state, the administration of glucose does not affect the turnover rate of phosphocreatine and adenosine triphosphate.

#### B. EFFECT OF INSULIN ON PHOSPHORUS TURNOVER IN MUSCLE

In the experiments of Sacks (152), five insulin units were employed per kg. cat weight. Half an hour after administration of labeled sodium phosphate to a cat by subcutaneous injection, 1.5 g. glucose was given per kg.; after the lapse of a further half-hour, insulin was administered. The cat was killed 105 minutes after injection of the tracer phosphate. The results of the experiment, shown in Table VI, indicate that both in resting and stimulated muscles insulin brings about an increase in the  $^{32}\text{P}$  content of phosphocreatine, adenosine triphosphate, and fructose-6-phosphate fractions. In the postabsorptive state, but not in the fasting state, insulin increases the  $^{32}\text{P}$  content of glucose-6-phosphate as well. Sacks suggests that in the latter case we are presumably concerned (see page 117) with an influx of glucose, in which nonintracellular  $^{32}\text{P}$  participates, and in the postabsorptive case with a renewal of the glucose-6-phosphate molecules, intracellular phosphorus participating.

A rather small amount of phosphorylation is observed in relation to the total probable glucose absorption. Two possible interpretations are given by Sacks: (1) the absorption of glucose by the muscle fiber does not involve the entry of a phosphate group into the cells, or (2) glucose-6-phosphate is not involved in the principal mechanism of glucose absorption by

TABLE VI

EFFECT OF INSULIN ON P TURNOVER IN MUSCLES OF CATS FED GLUCOSE (152)\*

State of animals	Phospho- creatine	ATP	Fructose-6- phosphate	Glucose-6- phosphate
Fasting, resting	103	77	66	514
Fasting, resting, given in- sulin	316	189	138	482
Fasting, stimulated and recovering	119	100	55	606
Fasting, stimulated and recovering, given in- sulin	271	205	203	733
Postabsorptive, resting	75	76	49	52
Postabsorptive, resting, given glucose	187	154	111	104
Postabsorptive, stimu- lated and recovering	113	103	66	70
Postabsorptive, stimu- lated and recovering, given glucose	203	156	120	116

\* Values are averages of several results and expressed as counts per minute per milligram P calculated on the basis of one million counts per minute injected per kilogram body weight. Time of experiments, 90 minutes.

the resting muscle (153). The first interpretation is in accordance with the results arrived at by Kjerulf-Jensen and Lundsgaard (108) in their study on phosphate exchange between plasma and muscle tissue with artificially perfused hind limb preparations in which labeled phosphate was employed. They found that both before and after addition of insulin the quantity of phosphate exchanging per unit time was slight in proportion to the simultaneously assimilated quantity of glucose. They therefore concluded that passage of glucose into the muscle cells in the form of hexose phosphate formed from the inorganic phosphate of the plasma was to be regarded as out of the question.

#### C. TURNOVER OF ACID-SOLUBLE PHOSPHORUS IN THE LIVER

The investigation of Kaplan and Greenberg (103) showed that the maximum  $^{32}\text{P}$  content of the free phosphate of liver tissue of fasting rats reaches its maximum 75 minutes after intraperitoneal injection of labeled phosphate, while the maximum activity of the total acid-soluble phosphorus is obtained after a lapse of 110–120 minutes (see Fig. 1).

The effect of dietary state of the animal on the distribution of acid-soluble  $^{31}\text{P}$  and  $^{32}\text{P}$  in the liver both of animals receiving only  $^{32}\text{P}$  and those receiving  $^{32}\text{P}$  and 300–400 mg. glucose as well is seen in Table VII.

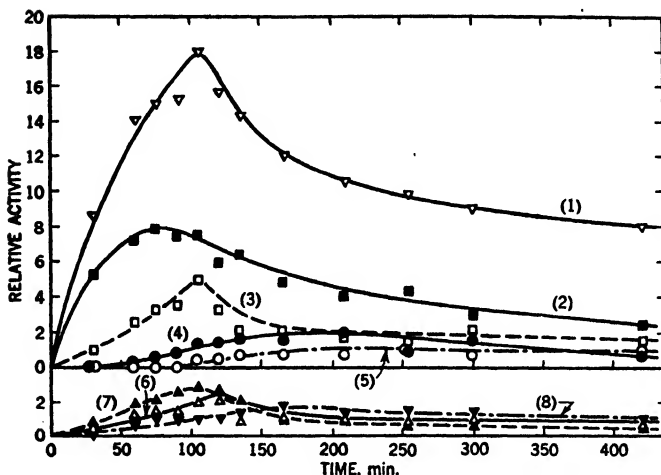


Fig. 1. Relative activity curves of liver phosphorus fractions of rats after administration of  $\text{Na}_2\text{H}^{33}\text{PO}_4$  (103). Curve 1, total acid-soluble P; 2, inorganic P; 3, alcohol precipitate; 4, labile P in adenosine triphosphate; 5, nonlabile P in adenosine triphosphate; 6, Hg precipitate; 7, residual P; 8, Pb precipitate. The points at 105 minutes are an average of ten determinations.

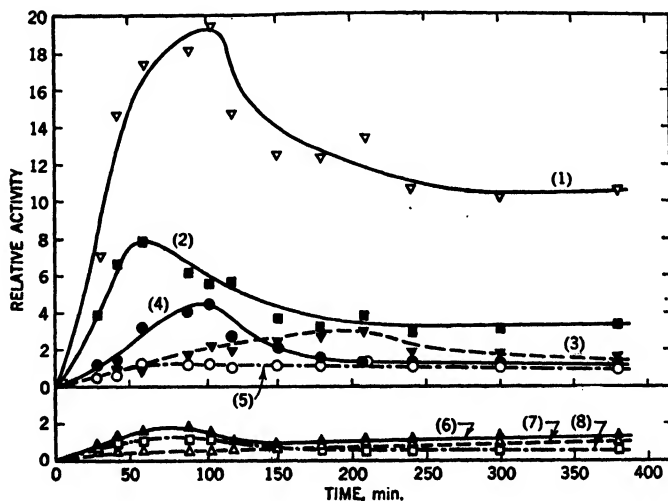


Fig. 2. Relative activity curves of liver phosphorus fractions of glucose-injected rats after administration of  $\text{Na}_2\text{H}^{33}\text{PO}_4$  (103). Identity of the curves is the same as in Figure 1. The points at 105 minutes are an average of eight determinations.

TABLE VII  
EFFECT OF DIETARY STATE OF ANIMAL ON DISTRIBUTION OF ACID-SOLUBLE  $^{31}\text{P}$  AND  $^{32}\text{P}$  IN LIVER (103)\*

Diet	No. of animals	Fraction of insoluble barium salts						Fraction of soluble barium salts					
		Total acid soluble		Inorganic		Labile P of ATP		Residual		Mercury ppt.		Alcohol ppt.	
		$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$	$\mu\text{P}$
Animals receiving only $\mu\text{P}$													
Controls (stock diet) fasted 12 hr. Fasted 72 hr. High carbohydrate High fat High protein	5	95.4 $\pm 1.9$	173.5 $\pm 6.4$	23.0 $\pm 0.67$	77.5 $\pm 3.5$	12.0 $\pm 0.46$	12.3 $\pm 0.77$	8.1 $\pm 0.37$	25.9 $\pm 2.3$	11.3 $\pm 0.44$	21.6 $\pm 1.3$	27.4 $\pm 0.98$	46.7 $\pm 2.1$
	6	83.2 $\pm 2.6$	206.8 $\pm 7.0$	27.7 $\pm 0.94$	138.6 $\pm 4.2$	7.4 $\pm 0.27$	10.0 $\pm 1.1$	8.0 $\pm 0.33$	13.4 $\pm 1.1$	9.9 $\pm 0.33$	13.7 $\pm 1.2$	21.3 $\pm 0.88$	22.0 $\pm 1.5$
	5	107.2 $\pm 1.7$	212.7 $\pm 4.5$	19.9 $\pm 0.42$	66.0 $\pm 2.9$	13.2 $\pm 0.83$	15.1 $\pm 1.3$	10.5 $\pm 0.55$	30.3 $\pm 1.5$	12.4 $\pm 0.48$	28.7 $\pm 2.0$	31.4 $\pm 1.2$	53.9 $\pm 2.9$
	5	80.6 $\pm 2.9$	143.2 $\pm 7.2$	22.9 $\pm 0.66$	88.6 $\pm 3.4$	5.7 $\pm 0.51$	10.1 $\pm 1.2$	8.8 $\pm 0.46$	18.1 $\pm 1.5$	8.8 $\pm 0.55$	15.3 $\pm 1.4$	21.9 $\pm 0.67$	26.5 $\pm 2.4$
	5	86.0 $\pm 0.79$	204.4 $\pm 5.9$	30.6 $\pm 0.60$	119.6 $\pm 4.3$	8.6 $\pm 0.23$	10.7 $\pm 0.63$	9.5 $\pm 0.65$	25.2 $\pm 3.2$	8.8 $\pm 0.50$	20.1 $\pm 1.7$	21.8 $\pm 1.1$	30.6 $\pm 2.0$
Animals receiving $\mu\text{P}$ and 400 mg. glucose													
Controls (stock diet) Fasted 72 hr. High carbohydrate High fat High protein	5	103.4 $\pm 2.0$	206.6 $\pm 5.5$	20.0 $\pm 0.72$	58.3 $\pm 3.3$	20.8 $\pm 0.45$	46.5 $\pm 2.3$	13.4 $\pm 1.2$	11.2 $\pm 0.74$	21.0 $\pm 0.80$	29.7 $\pm 1.2$	27.9 $\pm 2.1$	
	5	85.1 $\pm 1.8$	208.5 $\pm 8.3$	26.5 $\pm 1.0$	116.6 $\pm 7.0$	12.9 $\pm 0.75$	24.4 $\pm 1.3$	9.1 $\pm 0.39$	18.5 $\pm 1.2$	21.6 $\pm 0.96$	18.6 $\pm 1.2$	18.6 $\pm 1.2$	
	5	115.4 $\pm 2.2$	241.3 $\pm 6.8$	19.4 $\pm 0.75$	58.6 $\pm 2.3$	22.3 $\pm 0.59$	57.7 $\pm 2.5$	13.5 $\pm 0.98$	33.0 $\pm 1.5$	34.4 $\pm 1.3$	32.5 $\pm 2.1$	18.6 $\pm 1.6$	
	4	84.5 $\pm 2.0$	152.5 $\pm 6.2$	20.9 $\pm 0.64$	75.4 $\pm 3.3$	10.5 $\pm 0.52$	22.1 $\pm 0.89$	9.7 $\pm 0.87$	21.3 $\pm 2.3$	21.7 $\pm 0.67$	18.6 $\pm 1.4$	18.6 $\pm 1.4$	
	5	87.9 $\pm 1.2$	218.9 $\pm 4.2$	27.0 $\pm 1.2$	101.4 $\pm 4.3$	14.5 $\pm 0.62$	30.3 $\pm 2.1$	9.0 $\pm 0.71$	25.9 $\pm 1.8$	22.2 $\pm 0.86$	27.5 $\pm 2.0$	27.5 $\pm 2.0$	

\* All animals except those fasted 72 hours were fasted 12 hours before injection with the  $\text{NaHPO}_4$  containing  $^{32}\text{P}$ . All animals were sacrificed 110 minutes after receiving the injection. The values for  $^{31}\text{P}$  are in milligrams P per 100 grams fresh liver;  $^{32}\text{P}$  in parts per 1000 of the administered dose per 100 grams fresh liver. The measure of variability is the standard error of the mean.

The total acid-soluble  $^{32}\text{P}$  content of the liver is increased on all but the high-fat diet. In the fasted, fat-fed, and protein-fed rats a relatively large part of the  $^{32}\text{P}$  is present in inorganic form. These animals show a decreased ability to fix inorganic phosphate. The liver of the high-carbohydrate-fed animals shows a decrease in inorganic  $^{32}\text{P}$  content. These animals convert a larger percentage of the labeled phosphorus of the liver into organic form than do animals on any other diet.

It is apparent from examination of the curves of Figures 1 and 2 that injection of glucose causes a marked change in time pattern of liver phosphate metabolism. In contrast to the control animals a great deal of  $^{32}\text{P}$  is rapidly incorporated into the labile groups of adenosine triphosphate. Inorganic  $^{32}\text{P}$  disappears more rapidly in the glucose-treated animals than in the controls. Inorganic  $^{31}\text{P}$  also decreased as a result of introduction of glucose.

The effect of propanediol phosphate on the rate of renewal of the acid soluble phosphorus fractions in rat liver was investigated by Lindberg (116,117). For 20 days rats were fed a diet containing 100 mg. phosphorus as propanediol phosphate. To the rats weighing 55 g. labeled sodium phosphate was then injected intraperitoneally. After the lapse of one hour the acid-soluble phosphorus compounds of the liver were extracted. The specific activity of the phosphorus fraction obtained by acid hydrolysis for ten to twenty minutes, the "hexose ester" fraction, was found to be much higher than that of the corresponding fraction in the controls.

In tissue slices of rat kidney and liver the accumulation of organic  $^{32}\text{P}$  was found to increase appreciably when sodium fluoride was added to the medium containing labeled phosphate. This result is interpreted to be due to the inhibitory effect of fluoride on the phosphatase causing breakdown of the newly synthesized organic compound. The  $^{32}\text{P}$  was found to be present in the phosphoglyceric acid fraction. In the absence of oxygen accumulation of  $^{32}\text{P}$  in the organic fractions was found to be much reduced (104).

**Effect of Insulin on the Phosphorus Turnover in Liver.** Insulin and glucose administrations have a marked influence on acid-soluble  $^{32}\text{P}$  content of liver (103). Rats, weighing about 200 g., were fasted for a period of twelve hours, given the treatment shown in the table, and then injected intraperitoneally with trace doses of labeled sodium phosphate. The results obtained are seen in Table VIII.

The maximum in  $^{32}\text{P}$  concentration of the total acid-soluble phosphates was usually attained at about 110 to 120 minutes (see Figs. 1 and 2). The peak in radioactivity of



TABLE VIII

DISTRIBUTION OF LABELED PHOSPHORUS IN ACID-SOLUBLE PHOSPHORUS OF LIVER 110 MINUTES AFTER DISTRIBUTION OF THE PHOSPHATE (103)\*

Treatment	Total acid soluble	Inorganic	ATP		Alcohol soluble†	Residual†
			Labile	Non-labile		
Control, fasted 12 hr.	177	77.6	11.7	3.3	44.0	28.6
Glucose (300-400 mg. intraperitoneally)	204.0	56.6	49.0	12.8	25.4	12.9
Insulin	244.3	92.5	41.5	15.6	26.2	14.5
Glucose and insulin	262.0	83.4	64.5	16.2	22.7	20.2

\* All values are in per cent of administered dose  $\times 10$  of the labeled P per 100 grams fresh liver.

† This fraction consists largely of glycerol phosphate, but it contains also some hexose monophosphates.

‡ This fraction consists largely of phosphoglyceric acid.

the total phosphorus of ATP in the animals given glucose occurred at 110 minutes after the injection of  $^{32}\text{P}$ , whereas the peak in the control group occurred at 200 minutes. The peak in radioactivity of nonlabile phosphorus of the adenosine triphosphate was similarly shifted from 250 to 110 minutes. The peaks in the radioactivity of the alcohol and residual fraction were displaced in time by glucose administration from 110 to 210 and 245 minutes, respectively.

Phlorizin, malonate, and fluoride, but not iodoacetate, prevent a rise in the  $^{32}\text{P}$  content of liver following administration of glucose. These findings support the hypothesis that the primary action of phlorizin is a blocking of the formation of ATP.

Reduction of food intake causes a decrease in the acid-soluble phosphorus content of liver (134). The effect of insulin may therefore possibly partly or wholly be due, not to an increased rate of renewal of acid-soluble phosphorus molecules present in the liver, but to an increase in total ATP and free phosphate content following the administration of insulin.

In experiments on perfused cat liver, a high percentage of the ATP molecules and a minor percentage of the ester phosphorus were found by Lundsgaard to be renewed in the course of one hour (119).

Five minutes after intravenous injection of radioactive phosphate, Kalckar and his associates (102) found the labile phosphorus of adenylyl pyrophosphate present in rabbit liver to have a specific activity of 83% of the corresponding value for inorganic intracellular phosphorus. In interpreting this and similar figures, we must take into account that in the course of five minutes the plasma activity declines considerably, very highly active phosphate penetrating the liver cells in an early phase of the

experiment. When we calculate the percentage renewal from the ratio of the specific activity of the pyrophosphate phosphorus and that of the inorganic phosphorus at the end of the experiment, we obtain correspondingly too high a figure for the extent of renewal. Kalckar and his associates estimate the rate of rejuvenation of pyrophosphate phosphorus as 15–20  $\mu\text{g.}$  per minute per g. liver and consider these to be minimum values.

Rapoport and his colleagues (144) found the rate of renewal of labile phosphorus of adenosine triphosphate of kidneys to be reduced under the action of phlorizin. While in the controls the rate of renewal of labile phosphorus amounted to 70% in thirty minutes when phlorizin was previously administered by intravenous injection, the value was reduced to 33%.

#### D. EXPERIMENTS *IN VITRO* ON RATE OF RENEWAL OF ADENOSINE TRIPHOSPHATE

Experiments *in vitro*, in which much simpler conditions prevail than *in vivo*, were used for a calculation of the rate of renewal of the labile phosphorus in ATP. In determining the rate of interchange between free phosphate and pyrophosphate, Meyerhof and associates (125,127) studied the reaction: 2 triosephosphoric acid + 2 pyruvic acid + 1 adenosine-monophosphoric acid + 2 phosphoric acid  $\rightleftharpoons$  2 phosphoglyceric acid + 2 lactic acid + 1 adenosinetriphosphoric acid.

To a solution, kept at 20°C., containing dialyzed muscle extract, phosphoglyceric acid, hexose diphosphate, sodium lactate, pyruvic acid, adenosine triphosphate (containing 0.327 mg. pyrophosphate phosphorus), magnesium, manganese, sodium fluoride, and cozymase, labeled phosphate (containing 0.395 mg. phosphorus) was added. The distribution of labeled atoms between the free phosphate and the pyrophosphate fraction was then determined at different intervals. After the lapse of twenty seconds, as is seen in Table IX, almost half of exchange equilibrium of  $^{32}\text{P}$  between the two fractions was obtained. The average time of interchange of a pyrophosphate group with a phosphate group was shown to be fifty seconds.

It is quite possible that in muscle cells, where the enzyme concentration is ten times as large as in the experiments described above, a similar or even more rapid interchange takes place. The observation—that *in vivo*, after a lapse of several minutes, the interchange is somewhat below 100% (see page 115)—may be due to obstacles the labeled phosphate ions must overcome to reach the place of formation of ATP molecules. But it is also possible that the average rate of renewal of the ATP molecules in cells is smaller than that found in the system described above. Whether

TABLE IX

DISTRIBUTION BETWEEN INORGANIC AND PYROPHOSPHATE P OF 0.395 MILLIGRAM  
INORGANIC P ADDED TO AN *in Vitro* MUSCLE SYSTEM (127)

Time, sec.	Inorganic P, mg.	Pyrophosphate P, mg.	Interchange, %
0	0.395	0	0
20	0.313	0.084	47
40	0.288	0.120	64
75	0.253	0.135	77
150	0.255	0.144	85
600	0.223	0.159	92
	0.216	0.179	100

all ATP molecules present in the muscle cells behave in the same way—and the same question applies to other compounds as well—is a problem in the solution of which isotopic indicators may play an important part.

The renewal of phosphorus compounds goes hand in hand with the phosphorylation processes that are closely connected with oxidative steps in the utilization of carbohydrates. Each phosphorylation first involves adenosine phosphate and thus necessitates a renewal of the labile phosphorus of ATP molecules. Assuming two phosphorylations through the terminal group of ATP for every atom of oxygen, and taking the oxygen consumption to be 1.0 ml. per g. per hour, Furchgott and Shorr (63) calculate that, in 1 g. of cardiac tissue of the dog, 178.4 micromoles of phosphorylation takes place. This would represent a turnover of phosphorus, from intracellular inorganic P to the terminal group of ATP, of about 92  $\mu$ g. per g. tissue per minute. Assuming a rate of renewal in the resting muscle corresponding to that found by Meyerhof and his associates in their experiments *in vitro*, and taking 1 g. muscle to contain 120  $\mu$ g. terminal ATP phosphorus, the rate of turnover proves to be 144  $\mu$ g. per g. tissue per minute.

#### E. PHOSPHORUS METABOLISM IN BRAIN

Borell and Örström (24) investigated the uptake of  $^{32}$ P by different parts of the brain. The phosphorus present in the pineal body, the anterior and posterior lobes of the hypophysis, and the plexus chorioideus was found to interchange with the labeled phosphate administered by intraperitoneal injection at a much more rapid rate than the other parts of the brain (see page 143); the latter exhibit about the same slow phosphorus interchange as the cerebellum, as seen in Table X.

TABLE X

SPECIFIC ACTIVITIES OF TOTAL PHOSPHORUS OF BRAIN AREAS AND BLOOD OF RATS (24)\*

Part	Interval between injection of $^{32}\text{P}$ and killing of animal						
	40 min.	90 min.	2 hr.	2 hr., 20 min.	2 hr., 45 min.	24 hr.	24 hr.
Pineal body	820	520	1710	775	2080	...	...
Lobus anterior hypophysis	258	410	600	565	652	390	640
Lobus posterior hypophysis	234	355	290	492	440	250	425
Plexus chorioideus	930	615	975	1040	420	485	255
Lobus olfactorius	90	124	180	125	200	136	...
Lobus occipitalis	70	72	150	86	93	92	...
Lobus parietalis	78	106	60	43	93	...	...
Thalamus	42	75	45	43	...	32	100
Anterior tubercinereum	136	138	110	146	185	97	100
Posterior part of tubercinereum	63	69	115	82	180	97	100
Corpus mamillare	50	..	95	111	140	80	142
Substantia perforata	63	..	85	68	233	59	325
Corpora quadrigemina	63	..	65	71	100	40	115
Cerebellum	100	100	100	100	100	100	100
Pons	63	56	45	50	..	54	100
Medulla oblongata	48	73	72	64	..	97	85
Blood	4350	2920	1910	2800	2550	880	410

\* Specific activity of cerebellum = 100.

Of the  $^{32}\text{P}$  accumulated in the course of 40 minutes in the pineal body about 65% are present as acid-soluble phosphorus, 25% as inorganic phosphorus, and 10% as non-acid-soluble phosphorus.

Meyerhof (126) arrived at the result that the glucose molecule passes anaerobically through twelve stable intermediary stages before becoming alcohol and carbon dioxide (glucose metabolism in *Fusarium* is reported to show a different behavior, 136). At least three dissociable organic coenzymes, twenty or more enzyme proteins, and some bivalent metals (manganese and magnesium) are regarded as necessary to the breakdown (126). Since phosphate participates in almost every reaction, the application of labeled phosphorus in a detailed study of glucose metabolism may elicit information of interest. While, as described above, radiophosphorus was applied in the study of some phases of glucose metabolism, an extended application of this tracer in such studies has as yet not been made.

### III. Turnover of Phosphatides

#### A. GENERAL REMARKS

As was shown by early experiments, the presence of labeled phosphatides can be detected in the tissues shortly after administration of labeled

phosphate (14,67,141). The percentage of the dose administered present in the liver of the rat as phosphatide ( $^{32}\text{P}$ ) first increases, then declines after

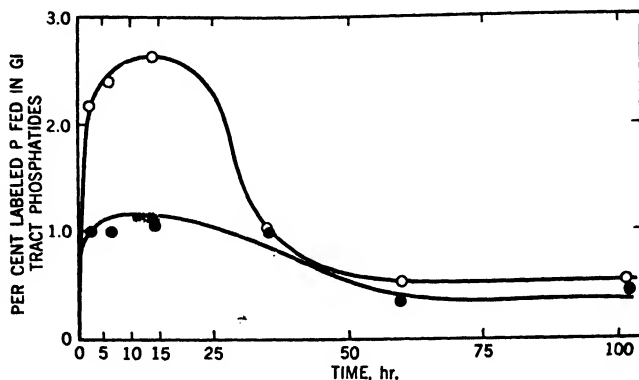


Fig. 3. Labeled phosphatide content of the gastrointestinal tract (141) obtained from rats fed 1 cc. cod liver oil along with 4 mg. of labeled P (O) and from rats that received 4 mg. of labeled P (●). Each point represents the average of four analyses on two rats.

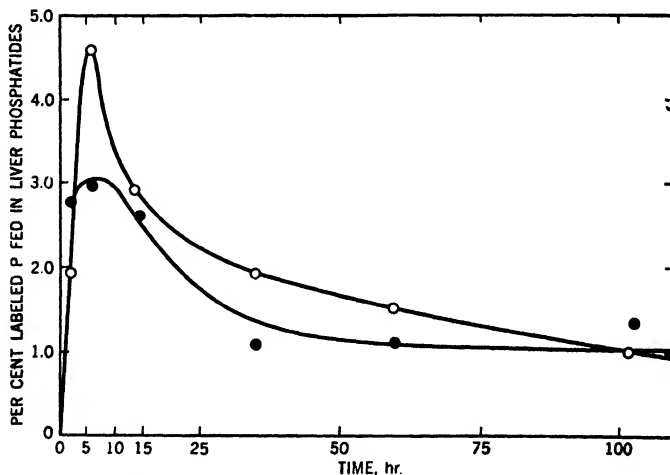


Fig. 4. Labeled phosphatide content of liver (141). Symbols have the same meaning as in Figure 3.

about ten hours, as is seen in Figures 3 to 7 taken from a publication of Perlman and associates (141). A similar behavior is shown by the labeled

phosphatide content of the gastrointestinal tract. The labeled phosphatide content of the carcass increases up to 100 hours.

The rapid increase in labeled phosphatide content of the liver indicates

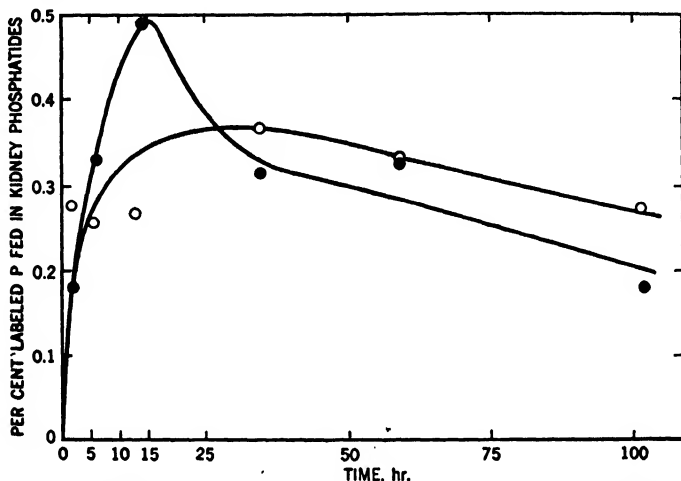


Fig. 5. Labeled phosphatide content of kidney (141). Symbols have the same meaning as in Figure 3.

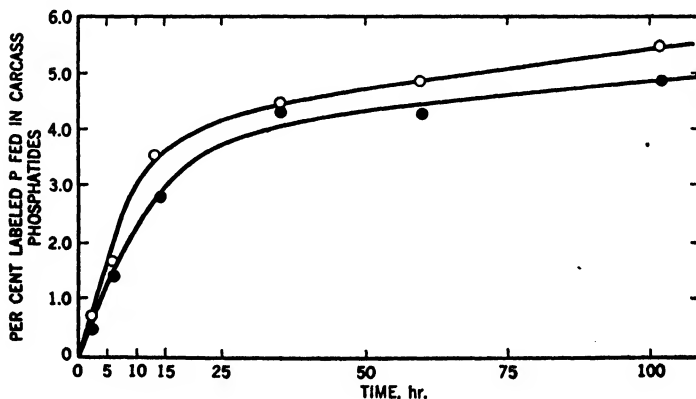


Fig. 6. Labeled phosphatide content of the carcass (141). Symbols have the same meaning as in Figure 3.

rapid turnover. In the early phases of the experiment, the specific activity of cellular phosphate is high and the newly synthesized phosphatide molecules incorporate highly active phosphate. The labeled phosphate mole-

cules in later phases of the experiment are repeatedly renewed with the participation of less active phosphate, and labeled phosphate makes its way in the later phases of the experiment from the liver into the plasma. This leads to a decrease in labeled phosphatide content of the liver. Apart from the high rate of renewal of phosphatide molecules, a high permeability of the liver cells to phosphate is responsible for the shape of the curve shown in Figure 4. Labeled phosphatides of the liver, furthermore, can enter

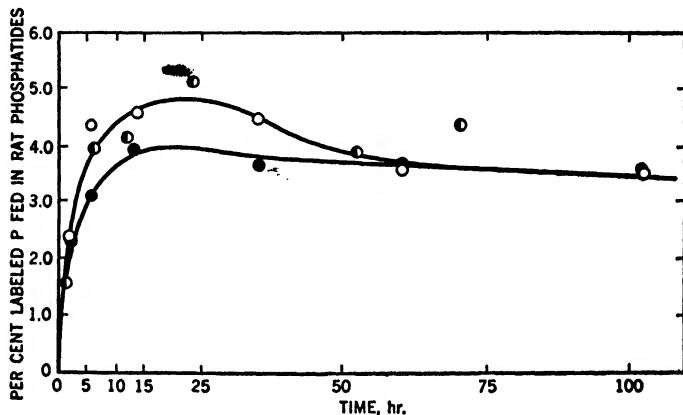


Fig. 7. Labeled phosphatide content of the whole rat (141) obtained from rats fed 1 cc. cod liver oil along with 1.5 mg. of labeled P (●), obtained from rats fed 1 cc. of cod liver oil along with 4 mg. of labeled P (○), and from rats fed 4 mg. of labeled P only (●). All points obtained by summation of the results of analyses of individual tissues.

the circulation, but the amount leaving the liver is only 1% of the phosphatide content of the liver per hour (see page 152). That the labeled phosphatide content of the muscles increases in the course of the first 100 hours (see Fig. 6) may be interpreted as due partly to the low rate of formation of phosphatide molecules in the muscle and partly to the low rate of penetration of phosphate into the muscle cell.

The above data indicate the formation of a labeled phosphatide mixture mainly composed of lecithins, cephalins, and sphingomyelins. The rates of formation of these constituents of the phosphatides vary, though the differences are, in most cases, as discussed on page 137, not very pronounced. The maximum activity of the total labeled phosphatide content of the rat is reached after the lapse of about 24 hours; while in the liver the most active organ, a maximum content is obtained about 10 hours after administration of labeled phosphate.

In the fasting mouse, Hodge and his associates (93) found the specific activity of all phosphatide fractions, with the exception of the  $\alpha$ -cephalin fraction, to increase appreciably. A maximum specific activity for the  $\alpha$ -cephalin was reached on the second fasting day.

The ultimate aim of investigation of the origin of phosphatide molecules in the body is the determination of the form in which the hydrogen, carbon, nitrogen, oxygen, and phosphorus atoms of phosphatide molecules have been taken up by the body and of the steps in which these atoms have been involved before their ultimate incorporation into phosphatide molecules. This exacting problem can hardly be solved at present, and we must be content with the determination of the sites and rates of formation of phosphatide molecules in the body from glycerol, fatty acid, choline (or other organic bases), and phosphate.

The lecithin molecule may be regarded as formed from five primary components: two fatty acids, glycerol, choline, and phosphoric acid. These are joined by two general types of bonds: the fatty acid-glycerol ester linkage and the phosphate-alcohol ester bond. Phosphate is bound in the lecithin molecule by two ester linkages, one of which is with glycerol and the other, with the base choline. It is conceivable that, after the phosphate group is split off the phosphatide enzymically, other ("new") phosphate radicals become combined with the dephosphorylated compound at a more rapid rate than the renewal of this compound takes place. In this instance the rate of renewal of the phosphate radicals will be greater than the rate of renewal of the other components of the molecule, and the turnover rate of phosphatide phosphate will supersede that of the fatty acids and nitrogen base present in the molecule. A combined investigation of the turnover rate of phosphatides, using labeled phosphorus, labeled nitrogen, and labeled hydrogen, may be expected to supply information regarding the relative rates of renewal of the different constituents of the phosphatide molecule.

If we make the assumption that phosphate ions or phosphorus-containing precursors which attain rapid equilibrium with the phosphate ions are incorporated into the phosphatide molecule, we can determine the rate of renewal of phosphatide molecules by comparing the specific activity of the phosphatide phosphorus at the end of the experiment with the mean specific activity of the intracellular inorganic phosphorus prevailing during the experiment. This calculation involves the further assumption that it is the intracellular, inorganic phosphorus which is incorporated into the phosphatide molecule. The rate of renewal of phosphatides extracted



from the liver of the rat and of the rabbit is calculated by comparing the specific activities of the phosphatide phosphorus and tissue inorganic phosphorus at the end of the experiment taking four hours, the result obtained being shown in Table XI (69). Since the average specific activity of cellular inorganic phosphorus of the liver in experiments taking four hours does not differ very much from the specific activity of cellular inorganic phosphorus measured at the end of the experiment and the difference between the specific activities of the intracellular and tissue inorganic phosphorus is in this case negligible (3), when computing the data of Table XI the tissue inorganic phosphorus values determined at the end of the experiment were considered.

TABLE XI  
RENEWAL OF LECITHIN AND CEPHALIN IN RAT AND RABBIT LIVER (69)\*

Fraction	Per cent renewal	
	Rat liver	Rabbit liver
Free P	100	100
Lecithin P	20.4	6.3
Cephalin P	18.6	6.0

\* Radiophosphorus administered four hours prior to killing the animal.

To obtain renewal figures that are certain to be correct, we should know the specific activity of the immediate phosphorus-containing precursor of the phosphatide molecule, and insofar as different paths of phosphatide formation exist, each involving a phosphorus-containing precursor, we should know the specific activity of all these precursors. Furthermore, if the site of formation is inside the cell, this site need not necessarily involve the whole cell but only part thereof. In the latter case, it is the specific activity of the immediate precursor in this part of the cell, not that of the whole cell, that is to be considered when calculating the rate of renewal. As is seen from the above considerations and from the more detailed discussion by Chaikoff (32), difficulties are involved in attempts to arrive at an exact measure of the rate of phosphatide turnover.

In the calculation of the renewal figures given in Table XI, the possibility of repeated renewal was disregarded. When a high percentage of the molecules is renewed, such a procedure is no longer permissible. A calculation of the rate of turnover has been carried out by Zilversmit and associates (184). Application of this calculation to the rate of formation

of labeled compounds is still lacking. The result was, however, applied in the calculation of the turnover rate and turnover time of labeled phosphatides introduced into the circulation, which is discussed on page 153.

The knowledge of exact turnover figures is of interest when comparing, for example, the amount of fat metabolized with the amount of phosphatides turned over. In many cases however we can draw conclusions merely by comparing the specific activity of the phosphatides present in an organ under varying conditions. Such a procedure is applied in the study of the effect of ingested fat on the rate of renewal of phosphatides, of the effect of choline, cholesterol, and other substances on the turnover of liver phosphatides, of the site of formation of the yolk, embryo, and milk phosphatides, and so on.

A comparison of turnover rate of phosphatides and of other compounds, not only in different organs but even in different parts of cells, is a problem of great importance, solved so far only in a special case: the rate of renewal of cytoplasm phosphatides has been compared with the rate of renewal of nuclear phosphatides, as described on page 169.

#### B. PURIFICATION OF PHOSPHATIDES

After administration of labeled phosphate, free phosphate and also some of the acid-soluble fractions of organs are often much more active than the phosphatide fraction. It is therefore of great importance to purify the phosphatides and free them from all other active fractions. Levene's classical method, which is based on the extraction of the mineral constituents by shaking an ether solution of phosphatides with a water solution of acetic acid, does not remove the labeled free phosphate. When, however, acetic acid is replaced by hydrochloric acid, an effective separation can be obtained (69). The effectiveness of this method of purification is seen from the figures of Table XII (9).

TABLE XII  
PURIFICATION OF SOLUTIONS OF PHOSPHATIDES IN ETHER CONTAINING  
ADDED INORGANIC  $^{32}\text{P}$  (9)\*

Number of purifications	Activity of ether solution	Activity of water solution
0	10,000	....
1	.....	8600
2	.....	60.4
3	.....	8.8
4	.....	2.6
5	5.5	2.0

\* 100 ml. ether solution shaken repeatedly with 300 ml. 0.1 N HCl solution for 20 minutes.

Shaking the ether solution of the phosphatides with ten drops of saturated disodium phosphate solution containing excess solid disodium phosphate was found also to be very effective in removing traces of radioactive phosphorus (62).

### *Separation of Choline-Containing and Non-choline-Containing Phosphatides*

Taurog and his associates (174) found that phosphatides are absorbed quantitatively from a petroleum ether solution by magnesium oxide; 0.6 g. magnesium oxide suffices to absorb 0.85 mg. of phosphatide phosphorus dissolved in 15 ml. petroleum ether. Use of 1.75 g. magnesium oxide is however preferable when choline-containing phosphatides have to be separated from non-choline-containing phosphatides. The separation is based on the elution of the choline-containing phosphatides by methyl alcohol.

The separation of choline-containing phosphatides from non-choline-containing phosphatides can however also be accomplished by first dissolving the phosphatides in methanol and then treating this methanol solution with magnesium oxide. The following procedure is used.

**Extraction of Phosphatides from Liver.** The weighed tissue was ground with sand in a mortar, transferred to a flask, and extracted with alcohol at 55–60°C. for two hours with occasional shaking. Approximately 150 cc. of alcohol were used for 20–25 g. of liver. The supernatant alcohol was decanted through filter paper and the residue extracted with a second portion of alcohol for one hour. The contents of the flask were then poured through the same filter paper and the two alcohol extracts were combined. The tissue residue was then extracted overnight with ether in a Soxhlet apparatus and the ether extract was added to the alcohol extracts. The combined alcohol-ether extracts were concentrated to a small volume (3–4 ml.). This was carried out in a hot water bath (55–60°) under reduced pressure and in an atmosphere of carbon dioxide. This concentrate was extracted with several portions of petroleum ether (b.p. 30–60°) and the petroleum ether extract made up to a desired volume by the addition of more petroleum ether.

A measured volume of a petroleum ether extract of liver containing approximately 6 mg. of phosphatide phosphorus was evaporated just to dryness under reduced pressure in a carbon dioxide atmosphere and the residue was dissolved in methanol. Under these conditions all phosphatides were dissolved by the methanol. This was shown by phosphorus and choline determinations. The methanol solution was made to 100 ml., and 25-ml. aliquots containing approximately 0.060 mg. phosphatide phosphorus per ml. were added to 1.75 g. magnesium oxide in 50-ml. centrifuge tubes. The mixtures were allowed to stand for 25–30 minutes with frequent stirring and then were centrifuged. The methanol was poured off and the magnesium oxide was washed twice with 25-ml. portions of fresh methanol. The combined methanol supernatants were made up to 100 ml. and aliquots were taken for phosphorus and choline determinations. The results are recorded in Table XIII, which shows that the separation by this procedure is just as

complete as when the phosphatides are first absorbed from a petroleum ether solution and then eluted with methanol.

TABLE XIII  
ADSORPTION OF LIVER PHOSPHATIDES OF EXPERIMENTAL ANIMALS DIRECTLY  
FROM METHANOL (175)

MgO used for ad- sorption, mg.	Phosphatide added to MgO			Phosphatide remaining in methanol			
	Choline, mg.	P, mg.	$\frac{\text{Moles choline}}{\text{moles P}}$	Choline, mg.	P, mg.	$\frac{\text{Moles choline}}{\text{moles P}}$	Choline phosphatide, %
Rat liver phosphatide							
1.0	3.51	1.51	0.59	3.49	0.99	0.90	99
1.75	3.42	1.49	0.59	3.36	0.88	0.98	98
1.75	3.42	1.49	0.59	3.25	0.85	0.98	95
1.75	3.42	1.49	0.59	3.30	0.91	0.93	96
Dog liver phosphatide							
1.5	3.56	1.55	0.59	3.38	0.93	0.93	95
1.75	3.56	1.55	0.59	3.22	0.86	0.96	90
1.75	3.56	1.55	0.59	3.25	0.88	0.95	91
1.75	3.46	1.51	0.59	3.14	0.82	0.98	91
1.75	3.46	1.51	0.59	3.13	0.82	0.98	91

One of the difficulties encountered was a cloudiness of the methanol solutions which appeared after centrifuging the magnesium oxide-methanol mixtures. The first methanol supernatant was usually clear, but the supernatants from the two methanol washings were usually cloudy. It was found that the presence of a small amount of sodium chloride in the methanol prevented the solution from becoming turbid after centrifugation and did not affect the recovery of choline or the choline-phosphorus ratio. For this reason methanol containing approximately  $5 \times 10^{-3} M$  sodium chloride is used for the two washings of the magnesium oxide.

### C. EFFECT OF INGESTED FAT ON RATE OF RENEWAL

Some time ago Artom and colleagues (14) showed that feeding of oil promotes the turnover of phosphatides. Some of their results are recorded in Table XIV.

The feeding of oil had the greatest effect on phosphatide metabolism in intestinal mucosa. A marked effect was also found in liver and kidney, but not in other organs of the rat. The increased phosphatide activity may be due either to the formation of additional phosphatide originating from the ingestion of oil, or to an accelerated rate of replacement of the nonactive

TABLE XIV

RATIO OF TOTAL AND SPECIFIC ACTIVITIES OF PHOSPHATIDE PHOSPHORUS OF RATS  
ON OLIVE OIL DIET AND ON CARBOHYDRATE DIET (14)\*

Organ	Ratio of total activities	Ratio of specific activities
Intestine	2	1.5
Liver	1.6	1.2
Kidney	1.3	1.1
Parenchymal organs, lungs, muscles, brain	1	1

\* Results shown were obtained four days after administering labeled phosphorus.

phosphatide molecules by newly formed active molecules, or to both of these effects (see below).

The effect of ingested fat on the activity of tissue phosphatides has also been investigated by Perlman and associates (141). They studied the labeled phosphatide content of organs at different intervals; some of their results are shown in Figures 3-7 (pages 128-130).

A marked effect of ingested oil on the formation of labeled phosphatides was also found to take place in stomach and large intestine, but the amount of active phosphatides formed per gram of stomach and large intestine was much smaller than that formed per gram of small intestine. This shows that the major part of the phosphatide turnover taking place in the digestive tract may be ascribed to the small intestine. The same applies to the digestive tract of the bird (46). It was also found that removal of tissue very active in phosphatide formation (*i.e.*, the gastrointestinal tract, and, to a minor extent, the kidneys) does not markedly influence new formation of phosphatides in the liver (19).

That the rate of renewal of phosphatides in liver is accelerated if the fat content of the circulation is increased was also shown in experiments on perfused cat liver (68). With normal blood, 1.5% of liver phosphatide phosphorus was found to be replaced by active inorganic phosphorus added to the blood as sodium phosphate in the course of 2.5 hours, while with lipemic blood 2.7% was renewed. Thus, the effect of ingested fat on the rate of renewal of phosphatides is very pronounced in those organs which, like the intestinal mucosa and the liver, play a predominant part in fat metabolism.

Schmidt-Nielsen (159) found that, one hour after  $^{32}\text{P}$  was administered by intramuscular injection to a rat, the specific activity of phosphatide

tide phosphorus extracted from the intestine was four times larger, after feeding 2.5 g. peanut oil by stomach tube, than the specific activity of phosphatide phosphorus of the resting intestine. The increased  $^{32}\text{P}$  content cannot be ascribed to the general increase in cell activity, because intestinal loops absorbing glucose did not synthesize phosphatides at a rapid rate but at the same low rate as nonabsorbing intestine. No increase was found in the total amount of phosphatides (about forty micromoles phosphatide phosphorus per gram intestine) present in the intestine during fat absorption. The newly formed phosphatide molecules must therefore either be transported away or split up again near the place of formation. Presumably the latter process takes place mainly. Poisoning with phlorizin does not decrease the rate of formation of phosphatides.

The enhanced phosphatide turnover in intestinal mucosa of the rat which follows feeding of fat may be interpreted as indicating the role of phosphatide turnover in fat absorption.

The total amount of  $^{32}\text{P}$  incorporated in the phosphatides of normal and rachitic rats was determined by Dols and associates (41). Rachitic rats were found to contain less total  $^{32}\text{P}$  but more phosphatide  $^{32}\text{P}$  than the controls investigated. The phosphatide phosphorus content of normal rats weighing 35–40 g. was found to be 11% of their total phosphorus content.

#### D. RELATIVE SPEED OF FORMATION OF VARIOUS PHOSPHATIDES

Chargaff (36) compared the specific activity of the lecithin phosphorus and cephalin phosphorus extracted from 250–300-g. rats at 19 to 43 hours after oral administration of labeled sodium phosphate. The rate of turnover of cephalin was found to be somewhat higher as seen in Table XV.

TABLE XV  
RELATIVE SPEED OF FORMATION OF LECITHIN AND CEPHALIN IN RATS (36)

Rat No.	Time, hr.	Phosphatide	Weight, mg.	Relative speed of formation
1	19	Lecithin	1063.0	100
		Cephalin	353.0	113
2	43	Lecithin	935.3	140
		Cephalin	258.0	151

Comparison of specific activities of lecithin and cephalin phosphorus extracted 24 hours after oral administration of labeled sodium phosphate indicates that lecithin extracted from intestinal tract and liver is somewhat

more active than the cephalin while the opposite behavior is shown by lecithin and cephalin of the brain. In normal liver, in experiments that lasted 24 hours, somewhat greater renewal figures were obtained for lecithin than for cephalin. Chargaff (36, see also 39) found the cephalin-lecithin ratio to be 0.8. Artom and his colleagues (14) conducted experiments in which olive oil and labeled sodium phosphate were administered to rats nine hours before they were killed, and they report the ratio to be about 0.6.

In rat carcinosarcoma 256, the specific activity of lecithin reaches a peak after 30 hours; cephalin attains its peak after 40 hours (74). The rate of renewal of sphingomyelin in liver is slower than that of the other phosphatide fractions. In kidney about the same rate of renewal is found for all the phosphatide fractions (95). The  $^{32}\text{P}$  of sphingomyelin of rat organs, except in brain and muscles, reaches a maximum eight days after administration. At the end of eight days, the specific activity of liver is 0.027 and the intestinal mucosa 0.010, while for skeletal muscle the specific activity is 0.3312, and for brain 0.3008 is found (76,94).

Hahn and Tyrén (69) found a somewhat higher specific activity for lecithin phosphorus than for cephalin phosphorus extracted from rat and rabbit liver. In experiments taking 24 hours, brain cephalin was found to be more active than the brain lecithin (39).

#### E. EFFECT OF LIPOTROPIC SUBSTANCES ON PHOSPHATIDE TURNOVER IN LIVER

The striking effect of lecithin upon the liver of a depancreatized dog (maintained with insulin) was found to be due to its content of choline, which reduces the fat content of the liver of depancreatized rats as well as of rats fed a diet rich in fat. The fact that the rate of formation of new phosphatide molecules in the liver is accelerated by the administration of choline, was established by Perlman and Chaikoff (139). Rats were fed for three days on a diet high in fat and low in protein; on the fourth day, half of each group were given 3 mg. of labeled phosphate and 30 mg. of choline chloride, simultaneously. The remaining half was given the labeled phosphate only. All animals were killed 4 hours after the administration of  $^{32}\text{P}$ . While the phosphatides in the livers of the controls were found to contain 2.23% of the  $^{32}\text{P}$  administered, the liver phosphatides of the choline-treated rats contained 2.92% (see also 137). Increased formation of labeled phosphatides was found to appear approximately 1 hour after choline ingestion, and its effect had disappeared about 10 to 12 hours later.

As distinct from choline, which promotes the formation of new phosphatide molecules in liver, cholesterol was found to have an opposite effect.

While the control diet consisted of 5 g. of unsalted butter to which was added 0.015 ml. of a standardized cod liver oil and 0.1 ml. of a vitamin B concentrate, the cholesterol diet contained the above constituents and added amounts of cholesterol varying from 250–400 mg. All rats were injected with labeled phosphate 26 hours after first receiving these diets, and their livers were removed 4 hours later. The liver phosphatides of the control rats contained, on the average, 2.9% of the  $^{32}\text{P}$  administered, while the liver phosphatides of the rats fed cholesterol contained 2.3%.

In another set of experiments, choline and cholesterol were fed simultaneously. Rats were fed the cholesterol diet for 30 hours; they then received 30 mg. of choline chloride by stomach tube, and labeled phosphate subcutaneously. In these experiments, 5.07% of the  $^{32}\text{P}$  administered was located in the liver phosphatides, while the corresponding figure for the control animals was only 2.81%.

Thus, combined feeding of choline and cholesterol clearly promotes the formation of new phosphatide molecules in the liver. All these cases are probably explained by an accelerated rate of renewal and not by an additional formation of phosphatide molecules. In this connection it is of interest that the phosphatide content of liver was found to be independent of dietary choline (34).

Friedlander and colleagues (58) observed furthermore that the additional amounts of radiophosphatides formed under the influence of choline do not long remain in the liver. They pass into the plasma and increase the specific activity of plasma phosphatides. A single feeding of 300 mg. choline chloride per kg. of body weight increases markedly the phosphatide turnover in the plasma, as seen in Figure 8. While after 12 hours the effect of choline is most pronounced, after the lapse of 96 hours the specific activity of the plasma phosphatides shows almost the same value as found in the controls.

The mechanism by which choline increases the specific activity of plasma phosphorus is not known. Since this occurs in the absence of a

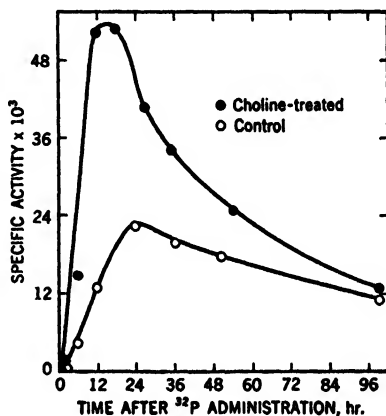


Fig. 8. Effect of choline on the specific activity-time relations of plasma phosphatide phosphorus in a dog (58).



change in the total phosphatide content of the plasma, it would appear that choline increases utilization of phosphatides. Such an increase in utilization of phosphatides could mean either (1) an increased transport of

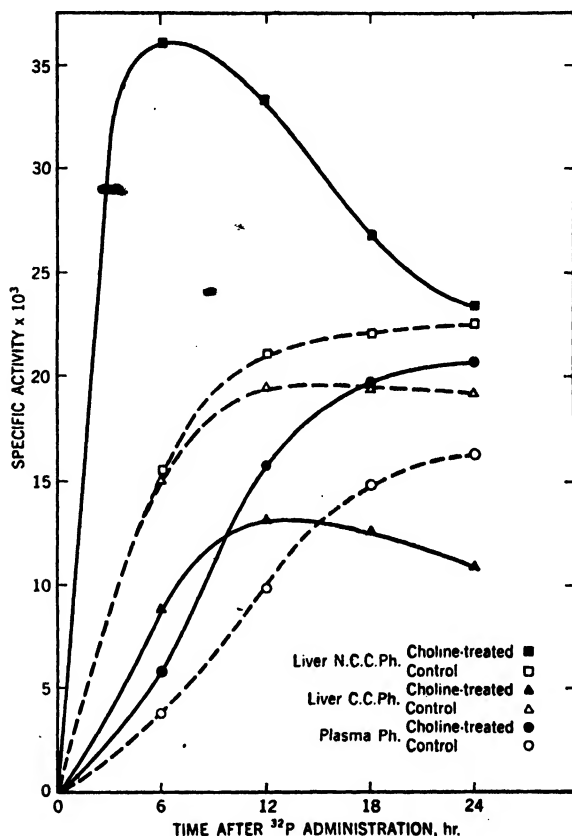


Fig. 9. Effect of choline on specific activity - time curve of choline-containing (C.C.Ph.) and non-choline-containing phosphatide (N.C.C.Ph.) phosphorus of liver (45).

phosphatides from liver to peripheral tissues or (2) an increased breakdown of choline-containing phosphatides within the liver.

Patterson and his associates found that choline deficiency resulted in decreased phosphatide turnover as well as in decreased concentration of phosphatides in liver (137) and kidneys (138) of rats. The fatty liver and

hemorrhagic kidneys which result from choline deficiency are apparently due to this decrease in phosphatide turnover.

Further evidence in support of the decreased rate of phosphatide turnover in choline deficiency is provided by studies (27) in which choline containing  $^{15}\text{N}$  was fed to both normal and choline-deficient animals. Incorporation of new choline (tagged by the presence of  $^{15}\text{N}$ ) was found to be retarded in the choline-deficient rat.

The following results were obtained by Entenman *et al.* (45) in comparing the effect of choline on the specific activity of choline-containing and non-choline-containing phosphatide phosphorus. The specific activity-time curves, as seen in Figure 9, of choline-containing and non-choline-containing phosphatide phosphorus of liver are quite similar in untreated dogs. A single ingestion of 300 mg. of choline per kg. body weight greatly increases, however, the specific activities of choline-containing phosphatide phosphorus of liver. Ingested choline does not increase specific activity of non-choline-containing phosphatide phosphorus of the liver. On the contrary, administered choline depresses its specific activity.

Specific activities of phosphatide phosphorus of various lobes of liver were compared in the experiments described. The values were found to be uniform: for the left main lobe, the right middle lobe, the left middle lobe, and the right main lobe, the values 0.014, 0.015, 0.015, and 0.014, respectively, were obtained.

The effects of a single dose of methionine, cystine, and cysteine upon the phosphatide activity of the liver of rats fed a high-fat and low-protein diet were also investigated (142). The amino acids were fed by stomach tube, simultaneously with injection of the labeled phosphate. The livers were analyzed eight hours after the injection. An increase of about 30% was observed in the rate of renewal of liver phosphatides of rats given the amino acids. Amino acids differ in their capacity to stimulate phosphatide activity in liver. Glycine, alanine, serine, tyrosine, proline, glutamic acid, and asparagine were found to increase the rate of renewal of liver phosphatides. A negative result was obtained with taurine, creatine, dihydroxyethyl sulfoxide, and sarcosine.

Choline phosphate inhibits turnover of phosphatides in liver. The inhibition appears to be limited to the noncholine phosphatide fraction. Choline phosphate, as a unit, is probably not utilized in the synthesis of phosphatides (147).

Ability of the organism to synthesize phosphatides is to a certain extent impaired by removal of a portion of the liver.

## F. TURNOVER IN KIDNEY

The specific activity of phosphatides extracted from rabbit and rat kidney is lower than that of phosphatides from small intestine and liver at early intervals after administration of radioactive phosphate. After the lapse of six hours the same result was obtained in experiments with dogs, but after eighteen hours the specific activity of the kidney phosphatide remained lower than that of liver and about equal to that of small intestine. At 98 hours the specific activities of the phosphatides in all three tissues were roughly the same (32). Acidosis induced by ingestion of ammonium chloride increases turnover of kidney phosphatides, according to Weissberger (181). Administration of phlorizin did not influence turnover rate of phosphatides in kidneys of the rat (182).

## G. TURNOVER IN MUSCLES

$^{32}\text{P}$  slowly appears as phosphatide phosphorus in skeletal muscle. This may be related to the slow rate of entry of labeled phosphate or of labeled phosphatides into muscle cells. The specific activity of phosphatides extracted from cardiac muscle, into which the phosphate penetrates at a higher rate than into skeletal muscle, is found to be higher (46). Evidence is, however, available that suggests a migration of labeled phosphatides from plasma into muscles. Artom (13, see also 59) injected radioactive phosphate into rats and cats in which the femoral and sciatic nerves of one leg had previously been cut. In the denervated muscle, newly formed phosphatides and, to a smaller extent, total phosphatides were found to be increased. The specific activity values of phosphatide phosphorus showed a gradient in the following order: liver > plasma > denervated muscle > intact muscle. The same gradient for the specific activities of the phosphatides was obtained in an experiment after introduction of a labeled emulsion of radioactive phosphatides. These results suggest that the labeled phosphatides synthesized by liver and released into the plasma penetrate the muscle cells, larger amounts probably being deposited in the denervated muscles (see page 152).

The turnover rate of phosphatides is found to be increased in muscle of rats maintained on a diet deficient in fat (91). In the fasting mouse, all phosphatide fractions, with the exception of  $\alpha$ -cephalin, which remained constant, showed a large increase in rate of renewal and reached a maximum on the second day of fasting (93).

## H. TURNOVER IN BRAIN

Brain is the organ in which both rate of penetration of labeled phosphate and incorporation of  $^{32}\text{P}$  into phosphatides is found to be lower than in any other organ; see Table XVI.

TABLE XVI  
FORMATION OF LABELED PHOSPHATIDES IN RABBIT BRAIN (86)

Duration of experiment	Ratio of specific activities of			
	brain inorganic P to plasma inorganic P	brain phosphatide P to plasma inorganic P	brain phosphatide P to brain inorganic P	brain phosphatide P to liver phosphatide P
250 min.	0.015	0.0002	0.016	0.005
11.5 hr.	0.030	0.0033	0.11	0.022
9 days	0.19	0.054	0.29	0.063
50 days	0.56	0.43	0.77	0.43

If and to what extent labeled phosphatides migrate from plasma into brain cells is not known. That labeled phosphatide molecules can be built up in brain tissue follows from experiments carried out with brain tissue slices, described on page 147.

The maximum  $^{32}\text{P}$  content (0.06% of the labeled phosphate administered per g. tissue) was observed in adult rat brain 200 hours after administration. In young brain, maximum content was observed only after 300 hours (33).

As the tissue formed in an organism given a labeled substance will necessarily become labeled, and, furthermore, as there is usually in the growing organism intensified enzyme action which leads to an accelerated rate of renewal of tissue compounds, we should expect a rapid new formation of phosphatides to take place in brains of growing rats. Rats of very different ages (including newly born rats) were studied by Changus *et al.* (33) and Fries *et al.* (60). The highest rate of formation of labeled phosphatides was found to take place at birth. Though this general characteristic was shared by all the parts investigated the phosphatide activity was by no means uniform throughout the nervous system; striking differences were encountered in the formation of labeled phosphatides in forebrain, cerebellum, medulla, and spinal cord, as seen in Figure 10.

From birth until the time the rat attains a weight of 50 g., a precipitous decline in active phosphatide content occurs throughout the central

nervous system of a rat born from parents to which  $^{32}\text{P}$  was administered previous to the birth of the rat. So striking is this loss in activity of brain phosphatides that, by the time the rat reaches a weight of 50 g., the spinal cord retains only 5% of the activity present in the newly born rat. During this period, the specific activity of plasma phosphate declines sharply, owing to a "dilution" of the  $^{32}\text{P}$  atoms of the plasma by nonactive phosphorus taken up with the food or given off by the organs. Active phos-

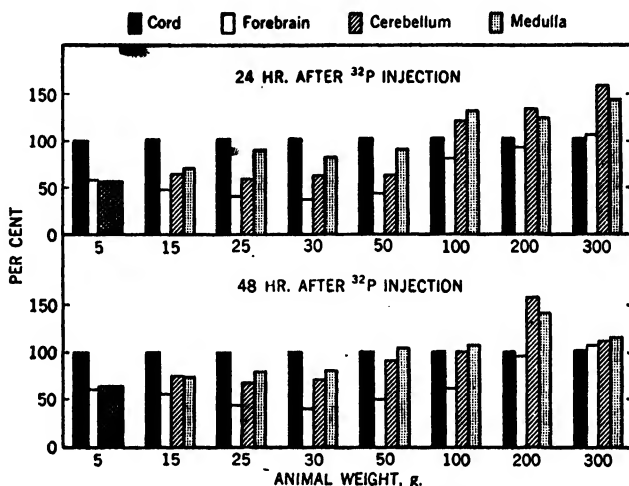


Fig. 10. Comparative phosphatide activities of forebrain, cerebellum, medulla, and spinal cord from birth until the time rat attains a weight of 300 g. (33). In each age group, the spinal cord has the arbitrary value of 100. Cross-hatched areas for the 5-g. rat represent values for medulla and cerebellum combined.

phatide molecules of the brain, when they become less active, are degraded and subsequently resynthesized with incorporation of less active inorganic phosphate. Thus, a considerable reduction in activity of brain phosphatides will indicate a high rate of renewal of phosphatide molecules of the central nervous system.

An abrupt change in rate of renewal of brain phosphatides occurs in the central nervous system of the rat during its growth from 30 to 50 g. As growth proceeds beyond 50 g., activity of brain phosphatides decreases throughout the central nervous system, but at a much lower rate than observed between birth and the age when the weight of 50 g. is attained. The spinal cord in the 200-g. rat possesses an activity of 20% of that of the 50-g.

rat, whereas in the 300-g. rat, the cord retains 15% of the activity found in the 50-g. animal. Forebrain, cerebellum, and medulla also lose activity as the animal grows from 50 to 300 g., but the rate of decline in activity is lower than that occurring in spinal cord (see Fig. 11). By the time a weight of 200 or 300 g. is reached, the relative activities of phosphatides forebrain, cerebellum, and medulla are as great as those of spinal cord, or even greater.

### I. ADRENAL GLANDS AND PHOSPHATIDE FORMATION

Ability of the adrenalectomized animal to synthesize new phosphatide molecules was established. The rate of formation of labeled phosphatide molecules in liver and small intestine of the rat is not influenced by complete removal of both adrenal glands (32,170a). Nor is the rate of incorporation of deuterium-containing fatty acids into the phosphatide molecules affected (20).

### J. TURNOVER IN NEOPLASTIC TISSUE

If the phosphatide molecules of carcinomatous tissue were replaced at a high rate by labeled molecules, and such molecules were given off by the tumor to the circulation shortly after the administration of labeled phosphate, the presence of carcinomatous tissue could possibly be diagnosed by determination of the activity of plasma phosphatides. The facts, however, that the phosphatide turnover of neoplastic tissue is much slower than turnover in liver and intestinal mucosa and that phosphatide molecules in the circulation were to a very large extent built up in the liver frustrate this possibility.

No appreciable difference is found in labeled phosphatide formation of spontaneous and transplanted tumors (80). In both cases, it is appreciably larger than that occurring in muscles, but smaller than that found in

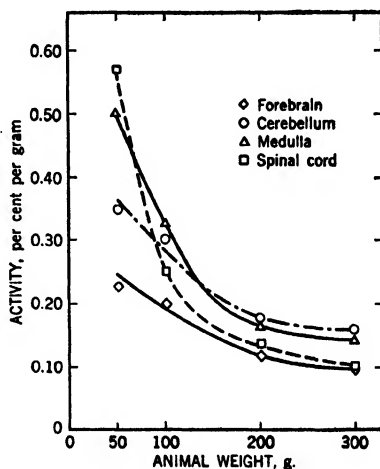


Fig. 11. Comparison of decline in phosphatide activity of forebrain, cerebellum, medulla, and spinal cord between the times the rat reaches weights of 50 and 300 g. (33). Symbols represent activities 24 hours after administration of labeled P.

liver. Thus, the formation of active phosphatide molecules in carcinomatous tissue is neither extremely pronounced nor very low.

A detailed investigation of phosphatide metabolism in mammary carcinoma, lymphoma, lymphosarcoma, and sarcoma 180 was carried out by Jones, Chaikoff, and Lawrence (99-101). Four tumors were transplanted into mice. They differed with respect to cell type and rate of growth. Two of the tumors produced metastases in distant parts, whereas the two others remained entirely localized at the place of inoculation. Uniform phosphatide activity was not found in the several types of tumor examined. The activity of lymphoma was only about one-third of that found in mammary carcinoma or lymphosarcoma. Cell type is apparently not a decisive factor in determining extent of phosphatide activity. Each tumor possesses a characteristic phosphatide turnover, which is independent of the host.

The maximum deposition of phosphatide  $^{32}\text{P}$  in neoplastic tissues may last from 10 to 50 hours.

In carcinosarcoma 256 the turnover of lecithin is somewhat more rapid than that of cephalin. The mode of behavior of the sphingomyelin fraction was found to be similar to that of cephalin but unlike that of the lecithin fraction of the same tumor (74). The specific activity of sphingomyelin phosphorus increases to a maximum at 48 hours after feeding labeled phosphate. Cephalin shows the same behavior in contrast to lecithin (95).

#### K: TURNOVER IN BLOOD

Shortly after administration of labeled phosphate, tagged phosphatides penetrate from the liver into the circulation. By 40 hours 0.5 to 0.1% of the administered  $^{32}\text{P}$  has been incorporated into phosphatides in

TABLE XVII  
SPECIFIC ACTIVITY OF INORGANIC AND PHOSPHATIDE PHOSPHORUS  
OF RABBIT PLASMA (86)

Time	Relative specific activity		Time	Relative specific activity	
	Inorganic P	Phosphatide P		Inorganic P	Phosphatide P
4 hours	100	0.53	45 hours	100	22.0
16 hours	100	3.8	55 hours	100	27.5
25 hours	100	8.1	9 days	100	816
37 hours	100	15.0			

the total plasma of the dog (50, see also 66). In experiments in which labeled inorganic phosphorus in rabbit plasma was kept at a constant level (86), the phosphatide phosphorus of the plasma showed the specific activities recorded in Table XVII. To what extent labeled phosphatide molecules are built up in the plasma can be investigated only by experiments *in vitro*. In such an experiment (66), lasting 4.5 hours, the specific activity of the phosphatide phosphorus was found to be less than 0.1% that of the inorganic phosphorus.

#### L. PHOSPHATIDE FORMATION IN TISSUE SLICES

The question whether a tissue can synthesize phosphatides independently or whether it acquires phosphatides from the plasma only after their formation by a more active tissue, was answered by Chaikoff *et al.* (62) in the following manner. A sciatic nerve of a dog stripped free of all adipose and connective tissue and weighing 300 mg. was placed in 5 ml. of Ringer solution containing radioactive phosphate. For control purposes the adipose-connective tissue surrounding the nerve was treated in a similar way. Conversion of radiophosphate from the Ringer solution into radiophosphatide by the nerve was found to be considerable, as is seen in Table XVIII. These experiments show that the nerve process, separated from the nerve cell body, can form phosphatides from inorganic phosphate.

TABLE XVIII  
FORMATION OF RADIOACTIVE PHOSPHATIDE BY DOG SCIATIC NERVES (62)\*

Time interval, hr.	Nerve	Adipose-connective tissue
0	0.006	0.008
0	0	0
4	0.63	0.036
4	0.72	0.026
4	0.44	...

\* All values are expressed as per cent labeled phosphorus of the Ringer solution incorporated into phosphatide per gram wet tissue.

Similar values were obtained for formation of radioactive phosphatide in brain slices of young and old rats. In the course of 4 hours 0.70 to 0.85% of the labeled phosphorus of Ringer solution was incorporated in brain slices (per gram wet tissue); in brain homogenate lower values (0.20 to 0.22) were obtained, as is seen in Table XIX.



TABLE XIX

## FORMATION OF RADIOACTIVE PHOSPHATIDES BY BRAIN (62)

Time interval, hr.	Brain slices						Brain homogenate					
	15-g. rat		50-g. rat		200-g. rat		15-g. rat		50-g. rat		200-g. rat	
	Wet	Dry*	Wet	Dry*	Wet	Dry*	Wet	Dry	Wet	Dry	Wet	Dry
0	0.0067	0.054	0.0058	0.028	0.0093	0.043	0.0047	0.038	0.023	0.11	0.027	0.13
0	0.0047	0.038	0.014	0.067	0.013	0.061	0	0	0	0	0.015	0.070
0	0.0067	0.054	0.0035	0.017	0.0096	0.045	0.0093	0.075	0	0	0.035	0.16
Average	0.0060	0.049	0.0078	0.037	0.011	0.050	0.00500	0.033	0.007	0.04	0.026	0.12
1	0.32	2.6	0.29	1.4	0.15	0.70	0.13	1.0	0.064	0.27	0.075	0.24
1	0.33	2.7	0.32	1.6			0.11	0.91	0.049	0.20	0.074	0.24
1	0.34	2.7	0.25	1.2	0.21	0.96	0.10	0.89	0.056	0.23	0.086	0.29
2	0.53	4.3	0.38	1.8	0.42	2.0	0.15	1.2	0.079	0.38	0.075	0.24
2	0.64	5.1	0.51	2.4	0.51	2.4	0.16	1.3	0.074	0.36	0.088	0.30
2	0.65	5.3	0.47	2.1			0.16	1.3	0.062	0.26	0.078	0.26
4	0.70	5.7	0.52	2.5	0.47	2.2	0.22	1.8	0.097	0.46	0.12	0.57
4	0.84	6.8	0.46	2.2	0.57	2.7	0.21	1.7	0.095	0.45	0.095	0.45
4	0.85	6.9	0.55	2.6	0.52	2.4	0.20	1.6	0.082	0.36	0.083	0.39

All values are expressed as per cent of labeled phosphorus of the bath incorporated into phosphatide per gram of tissue. All values recorded for 1, 2, and 4 hours have had the average zero-time value subtracted.

\* The water content of the forebrain of 15-, 50-, and 200-g. rats is 87.6, 79.2, and 78.6%, respectively.

This type of phosphorylation can only be detected by making use of labeled phosphate. The usual methods of chemical analysis fail to detect the synthesis of the small percentage of new phosphatide molecules, as the formation of these goes hand in hand with the autolysis of a comparatively large percentage of phosphatide molecules present at the start of the experiment. The amount of phosphatide found in brain homogenate after 4 hours is 10–15% less than that present at zero time.

Conversion of labeled, inorganic phosphorus into phosphatide phosphorus by surviving brain slices is greatly increased (up to about five times) by the addition of hexose, glucose, galactose, mannose, and fructose to the bicarbonate-Ringer solution containing labeled phosphate, as is seen in Figure 12. This increase in formation of labeled phosphatide could be due either to an increased rate of penetration of labeled phosphate into the site of synthesis of phosphatides, or to an enhanced rate of formation of labeled phosphatide molecules. That the presence of hexoses accelerates the rate of formation of labeled phosphatide molecules is shown by the following experiment. Brain slices, after being kept in a bicarbonate-Ringer solution containing  $^{32}\text{P}$ , are washed and placed in an inactive bicarbonate-Ringer

solution for two hours. While in the glucose-free Ringer solution hardly any further formation of labeled phosphatides takes place, a threefold increase of labeled phosphatide content is observed when the Ringer solution contains glucose.

It is well known that the oxygen consumption of brain preparations remains nearly constant for long periods when the preparations are placed in a Ringer medium containing glucose, whereas in a glucose-free medium the oxygen uptake decreases rapidly. Presumably the formation of labeled phosphatide molecules is promoted by increased oxygen consumption which provides increased oxidative energy for formation of phosphatide or of a phosphorus-containing phosphatide precursor. The stimulatory effect of the hexose upon formation of radiophosphatide does not occur under anaerobic conditions. The stimulation is abolished when the tissue organization is disrupted by homogenization. Addition of pentoses fails to increase the yield of radiophosphatides (158).

In both liver and kidney slices, phosphatide formation is greatly impaired in the absence of oxygen, as is seen in Table XX. Phosphatide formation in surviving liver and kidney slices is extremely sensitive to the presence of sodium cyanide. Concentrations as low as 0.001 *M* sodium cyanide inhibit the formation in liver slices to the extent of about 90%, as is seen in Table XXI.

Azide and hydrogen sulfide were found to have an effect similar to that shown by cyanide on formation of labeled phosphatides in tissue slices. Carbon monoxide was furthermore shown to have an inhibitory effect, which is more pronounced in the dark than in the presence of strong light (see Table XXII, also Taurog *et al.*, 1975).

Homogenized liver tissue completely loses its ability to incorporate phosphate into the phosphatide molecule (51). Much less phosphatide formation is shown by homogenates of kidney and brain (see Table XIX,

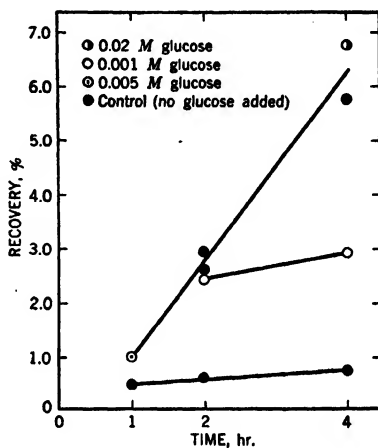


Fig. 12. Effect of various concentrations of glucose on recovery of radiophosphatide in brain slices at various time intervals (158).

TABLE XX  
EFFECT OF ANAEROBIC CONDITIONS ON PHOSPHATIDE FORMATION (176)

Rat No.	Period of incubation, hr.	Per cent added <sup>32</sup> P recovered as phosphatide per g. tissue				Inhibition, %
		Control, oxygen present		Anaerobic conditions		
		Wet weight	Dry weight	Wet weight	Dry weight	
Liver						
1	4	9.2	40	0.60	2.6	93
2	4	6.5	29	0.53	2.3	92
3	2	3.2	14	0.44	1.9	86
4	2	3.8	17	0.21	0.92	94
Kidney						
5	4	4.7	26	0.058	0.32	99
6	4	3.4	19	0.41	2.3	88
7	2	3.3	18	0.17	0.95	95
8	2	3.5	20	0.80	4.5	77

TABLE XXI  
EFFECT OF CYANIDE ON PHOSPHATIDE FORMATION  
DURING TWO-HOUR INCUBATION (129)

Rat No.	Cyanide, concentration, <i>M</i>	Per cent added <sup>32</sup> P recovered as phosphatide per g. tissue				Inhibition, %
		Control, cyanide absent		Cyanide present		
		Wet weight	Dry weight	Wet weight	Dry weight	
Liver						
1	0.03	1.6	7.0	0.065	0.29	96
2	0.03	2.4	11	0.011	0.048	100
3	0.01	1.7	7.5	0.098	0.43	94
4	0.01	2.2	9.7	0.015	0.066	99
5	0.01	2.2	9.7	0.057	0.25	97
6	0.01	2.2	9.7	0.29	1.3	87
7	0.01	3.0	13	0.14	0.62	95
8	0.005			0.054	0.24	
9	0.003	2.1	9.2	0.24	1.1	89
10	0.003	2.9	13	0.25	1.1	92
11	0.001	3.2	14	0.32	1.4	90
11	0.001	3.2	14	0.39	1.7	88
Kidney						
12	0.01	2.3	13	0.00	0.00	100
13	0.01	1.9	11	0.00	0.00	100
14	0.005	1.8	10	0.00	0.00	100
15	0.005	1.5	8.4	0.00	0.00	100

TABLE XXII

EFFECT OF LIGHT ON CARBON MONOXIDE INHIBITION OF PHOSPHATIDE FORMATION  
IN LIVER SLICES INCUBATING TWO HOURS (174)

Rat No.	Per cent added <sup>32</sup> P recovered as phosphatide per g. tissue						Inhibition, %	
	Control		CO in light		CO in dark			
	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	CO in light	CO in dark
1	1.9	8.4	1.4	6.2	1.1	4.8	26	42
2	1.5	6.6	0.87	3.8	0.59	2.6	42	61
3	2.1	9.2	1.1	4.8	0.68	3.0	48	68

page 148) than by slices of these tissues. The loss or decrease in activity of disintegrated tissue may be due to dilution of substances essential for the synthesis or to their destruction by enzymes (32).

One may be tempted to interpret the formation of labeled phosphatides in tissue slices as due to partial reversibility of hydrolysis of phosphatides. Even though the tendency of the reaction is far in the direction of decomposition, the reverse reaction may occur to a slight degree even during the early period of forward reaction. The results obtained by Chaikoff and associates with respiratory inhibitors exclude, however, the possibility that the formation of labeled phosphatides is due to a reversal of the decomposition of phosphatides proceeding in the tissue slices. In surviving rat kidney and liver slices much more radioactive organic phosphorus is found when the uptake of  $^{32}\text{P}$  is investigated in the presence of fluoride (104).

#### M. PATH OF CONVERSION OF INORGANIC PHOSPHATE TO PHOSPHATIDE

The path of conversion of inorganic phosphate to phosphatide is not known. Glycerophosphate, diglycerides, neutral fat, choline phosphate, phosphoproteins, or other compounds may be involved as intermediates. That aminoethylphosphoric acid can be excluded from this group of substances follows from the work of Chargaff and Keston (38). Experiments in which 80 mg. of labeled disodium aminoethyl phosphate was administered to adult rats by subcutaneous injection showed that the body was unable to utilize aminoethylphosphoric acid as such for the synthesis of cephalin. Of the  $^{32}\text{P}$  administered as aminoethylphosphoric acid, 28% was found to be excreted through the kidneys in the course of eight hours in these experiments.

Enzymic hydrolysis of aminoethylphosphoric acid in the tissues is presumably followed by utilization of the inorganic phosphate for synthesis of lecithin and of demethylation of lecithin to form cephalin. The aminoethylphosphoric acid normally occurring in the body tissue may be a product of catabolism of cephalin.

Glycerophosphate and phosphoryl ethanolamine containing  $^{32}\text{P}$  were prepared by Chaikoff *et al.* (32), and their incorporation into phosphatides of liver and kidney was demonstrated by surviving slices and also in the intact animal. These experiments gave no proof that breakdown of these labeled compounds to inorganic phosphate did not occur before conversion of the radioactive phosphorus to phosphatide.

#### N. RATE OF INTERCHANGE OF PLASMA PHOSPHATIDES WITH TISSUE $^{32}\text{P}$ PHOSPHATIDES

Rate of interchange of plasma phosphatides with tissue phosphatides was determined in experiments in which part of the plasma of a rabbit was replaced by an equal volume of plasma from another rabbit containing labeled phosphatides (81); similar experiments were also carried out with chicks (81) and with dogs (185). In another investigation an emulsion of phosphatides prepared from rats liver was introduced into the circulation of a rat (75). In the course of 10 hours a substantial part of the labeled phosphatide molecules were found to have left the plasma and were detected in different organs, especially in the liver. Some results obtained in experiments with dogs (185) are seen in Table XXIII and in Figure 13. The greater slopes of the curves of the figure found during the early phases

TABLE XXIII

DISTRIBUTION OF PHOSPHATIDES IN TISSUES OF THE DOG AT END OF FIVE HOURS (185)

Tissue	Injected dose per whole organ, %	Labeled phosphatide P per whole organ, mg.	Organ phosphatide supplied by plasma per hr., %
Plasma	53.0	18.5*	..
Liver	11.1	173	1.09
Kidney	1.13	28.6	0.67
Small intestine	2.44	68	0.61
Spleen	0.35	8.5	0.71
Red corpuscles	1.15	....	..
Muscle	4.2	....	..

\* Milligrams phosphatide P per 100 milliliters.

may be explained by supposing a nonuniform distribution of injected radio-phosphatide in the plasma during this interval. Zilversmit and colleagues

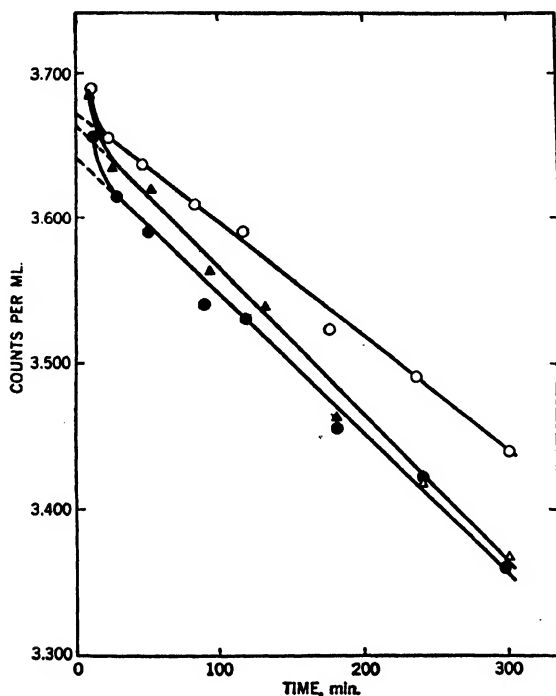


Fig. 13. Disappearance of labeled phosphatides from plasma of three different dogs (185).

calculate (184) the turnover time ( $T_t$ ) of the phosphatides by the formula derived from the slope of the curve in Figure 13.

$$T_t = - \frac{\log e}{\text{slope}} = - \frac{0.434}{\text{slope}}$$

and the turnover rate,  $p$ , from the turnover time and the total phosphatide content,  $r$ , of the plasma:

$$p = r/T_t$$

Labeled phosphatide found in liver and other organs originated almost

exclusively from the plasma, though the plasma injected contained, beside labeled phosphatide, an appreciable amount of free labeled phosphate as well. Of the 2,540,000 radioactive units, 600,000 were present in compounds other than phosphatides. (Three millicuries active phosphate was injected into the dog 26 hours before the plasma transfusion.) The amount of labeled phosphatides formed from free phosphate after transfusion of labeled plasma can almost be disregarded, as in 5 hours only 0.01% of the injected organic  $^{32}\text{P}$  was converted to phosphatide phosphorus per gram liver. Of the 14,400 counts of phosphatide phosphorus found per gram liver, only about 60 counts were thus synthesized in the liver.

During the postabsorptive phase 5.2 to 8.0 mg. phosphatide phosphorus is turned over per hour in the plasma of dogs weighing 6-9 kg. In experiments with rabbits, after the lapse of 4 hours, 29-38% of the labeled phosphatide molecules originally present in the plasma were found in the liver (81). In the course of 17 minutes, 65% of labeled phosphatides originally present in the plasma of the chick left the circulation (81).

It is in the liver that the phosphatide interchange takes place at the highest rate. The liver is thus not only the main organ in which phosphatide molecules are built up (see also page 138), but also that showing the greatest permeability to phosphatide molecules.

The fact that 76-83% of the injected labeled phosphatides can be accounted for in the seven tissues examined suggests that the breakdown of phosphatides in the animal tissue cannot be a rapid process.

That beside being the principal tissue in the body concerned with the synthesis and supply of plasma phosphatides the liver is also mainly responsible for the removal of phosphatides is shown by further work of Entenman and his associates (45a). When comparing the rates at which the injected labeled plasma phosphatides disappeared from the plasma of the normal and the liverless dogs, the rate of disappearance from the plasma of the normal dog was found to be six to ten times larger than the rate of disappearance from the liverless dog. The specific activities of the plasma phosphatide phosphorus of dogs that have received intravenously radioactive plasma phosphatides did not decrease significantly after the liver had been excluded from the circulation. In contrast to this result the labeled phosphatides disappeared at a normal rate from the plasma when the gastrointestinal tract was removed. While in normal dogs weighing from 7 to 18 kg. the plasma phosphatides are completely turned over in 6 to 10 hours, by depriving these dogs of their livers the time required for complete turnover was prolonged to 33 to 160 hours.

## O. PASSAGE OF PHOSPHATIDES INTO LYMPHATIC CHANNELS

As mentioned above, in the course of few hours a very appreciable part of the phosphatide molecules originally present in the plasma are found to be replaced mainly by phosphatide molecules previously present in the liver. Reinhardt *et al.* (146) found that part of the labeled phosphatide molecules injected into plasma reached lymphatic channels and were recovered in the thoracic duct lymph. It is not established through which tissues phosphatides migrate, although liver presumably plays an important part in this process. Some figures for the recovery of injected labeled plasma phosphatides are seen in Table XXIV.

TABLE XXIV  
RECOVERY OF INJECTED PLASMA RADIOPHOSPHATIDE IN THORACIC DUCT LYMPH  
OF A DOG (146)\*

Interval after injection of plasma, min.	Lymph			Plasma	
	Total lymph collected, ml.	Rate of flow, ml./min.	Phosphatide <sup>32</sup> P expressed as radioactive counts/min.		Phosphatide <sup>32</sup> P ex- pressed as radioactive counts, min./ml.
			Per ml.	For total sample collected	
Before	9.9	0.49			
4 before to					
40 after	23.9	0.54	94	2250	...
40- 90	26.4	0.53	216	5680	...
90-153	23.3	0.37	251	5850	937
153-214	23.9	0.39	213	5100	806
214-244	13.4	0.45	191	2560	...
244-272	13.3	0.47	191	2540	...
272-319	13.2	0.28	167	2220	...
319-359	13.6	0.34	178	2420	504

\* Dog weight, 17.9 kg.

The dog received intravenously 100 ml. of plasma containing a total of 759,200 counts per minute as phosphatide. The first sample of lymph was obtained during the first 36 minutes after the injection of radiophosphatide. This sample already contained over 2000 counts per minute. If we assume that plasma represents 5% of the body weight, the plasma still contained a total of 452,000 counts per minute at the end of this period of observation. Approximately 9% of the phosphatides that left the plasma was recovered from the lymph of the thoracic duct in the course of six hours and still higher figures (20%) were obtained in experiments with other dogs. Thus thoracic duct lymph serves as a medium for the return to the plasma of a significant fraction of the phosphatides lost from the plasma.



## P. STUDY OF LECITHINEMIA

About two hours after administration of a meal containing fat, the fat content and phosphatide content of the blood begins to rise. A maximum is reached after four hours (22). The increase in lecithin content of the plasma could be due to lecithin synthesized in the intestinal mucosa and absorbed into the blood or to mobilization of phosphatides synthesized in the liver or other organs.

The following experiment (89) shows that at least a large part of the phosphatide excess found in lipemic blood is not due to phosphatide molecules taken up from the intestine. At the start of an experiment 150 mg. labeled phosphorus as sodium phosphate was administered to a fasting dog weighing 7 kg., and another 150 mg. was given after the lapse of two hours. Simultaneously with the second sodium phosphate dose, 50 g. olive oil was fed. Six hours after the start of the experiment a pronounced lipemia was found to have taken place, and a rise of the blood phosphatide phosphorus amounting to 2.5 mg. per cent was observed in this lipemic state. Of this 2.5 mg. per 100 ml., however, only 0.048 mg. per 100 ml. was labeled phosphatide phosphorus. The rest was nonlabeled phosphatide phosphorus mobilized by the liver or by other organs.

When interpreting these figures, it must be considered that the intestinal tract of the fasting dog contained endogenous phosphorus which "diluted" the labeled phosphorus fed to the dog, and, furthermore, that an exchange of plasma phosphatides with organ phosphatides takes place which will result in the replacement of a part of the labeled blood phosphatides by unlabeled organ phosphatides. But it was found that in the course of two hours less than half the plasma phosphatides was replaced by organ phosphatides (Table XXIII, and ref. 81); thus renewal of labeled blood phosphatides during the experiment cannot explain the large difference found between the increment of total blood phosphatides and the increment of labeled blood phosphatides, the ratio of which was as high as 31. Nor can the effect of the "dilution" by intestinal phosphate of the labeled phosphate fed to the animal explain more than part of the above-mentioned difference.

In connection with the above consideration, it is of interest to consider some results obtained by Cavanagh and Raper (31). They found that the glycerides extracted from the plasma of rats six hours after feeding of deuterium-containing linseed oil contained ten times as much deuterium (in atoms per cent) as the phosphatides of the plasma. This result is understandable if most of the phosphatide molecules present in the plasma have been synthesized in the liver and not taken up from the intestine.

### Q. ORIGIN OF PHOSPHATIDES AND OTHER PHOSPHORUS COMPOUNDS OF YOLK

Prior to the use of labeled phosphorus, the question of site of formation of yolk material was far from settled, as will be seen from the following passage from a review of egg formation of the domestic fowl published in 1938 in *Physiological Reviews* by Conrad and Scott.

"Little is known of the mechanism of formation of this yolk material. There are three general ways in which yolk might be formed. The materials of the yolk might be synthesized elsewhere, carried into the ovary by the blood and deposited in the yolk. They might be formed in the follicular epithelial cells and secreted in the yolk. Finally, the yolk material might be synthesized in the yolk from nutrients reaching it through the follicular epithelium from the blood. Perhaps all three mechanisms have some part in the yolk formation."

A hen laying daily incorporates about 1.5 g. of phosphatide in the yolk, which corresponds to about 10% of the yolk weight. This percentage is much more than the amount contained in the daily food of the hen. Furthermore, it was found by different investigators that the fact that a fowl was raised on diets containing phosphorus in inorganic form only did not unfavorably influence its egg-laying capacity. It is, therefore, wholly or mainly in the organs of the hen that the synthesis of the phosphatide molecules of the yolk takes place. For the purpose of securing information regarding the organ in which the yolk phosphatide is primarily synthesized, experiments were carried out with  $^{32}\text{P}$  as an indicator (85).

Labeled sodium phosphate was administered to laying hens, the eggs laid were collected, and the specific activities of the phosphatide phosphorus extracted from the yolks were determined. In other experiments, the hen was killed and the specific activities of the phosphatide phosphorus of the yolks, ovary, liver, intestinal mucosa, and plasma were compared. The following figures show the results obtained for the specific activity of phosphatide phosphorus extracted from different organs of a hen 5 hours after administration of labeled sodium phosphate (85):

Organ	Relative specific activity
Liver .....	100
Plasma .....	.79
Ovary .....	7.2
Yolk .....	9.2
Intestinal mucosa .....	18

Ovary phosphatides are only slightly active; plasma phosphatides show a marked activity; and liver phosphatides show the greatest activity.

Thus, the gradient in flow of labeled phosphatides is directed from plasma to ovary. The explanation suggests itself that yolk phosphatides are supplied by plasma phosphatides and that the role of the ovary, in supplying egg phosphatides, is to remove phosphatides from plasma and to incorporate them into yolk. Nature endowed plasma of birds actively engaged in egg laying with a much higher phosphatide content (about 25 mg. per cent) than is found in plasma of other animals or in plasma of male birds and immature females, obviously in order to facilitate passage of the large amounts of phosphatides which the plasma of the laying bird has to carry into the ovary. The total phosphatide content of the plasma of the laying hen in question amounted to 15 mg. The hen, laying daily, incorporated about 50 mg. phosphatide phosphorus into the yolk within 24 hours (nearly four times the phosphatide content of the plasma). The phosphatide content of the plasma was thus almost wholly renewed in the course of the 5 hours of the experiment.

The figures on page 157 suggest, furthermore, the probability that phosphatide molecules of yolk are mainly synthesized in liver and are passed on by plasma to their destination. The liver contained 34 mg. of phosphatide phosphorus, and, since in the course of five hours some 9 mg. of phosphatide phosphorus was carried into the ovary, about one-fourth the liver phosphatides must have been renewed within 5 hours to supply the phosphatides incorporated into the yolks, a figure compatible with the results obtained in the investigation of rate of renewal of liver phosphatides (see page 132). The fact that the yolks show but a small activity is due to the dilution of the strongly active phosphatides incorporated during the last 5 hours by the large amounts of nonlabeled phosphatides already present in the yolk.

Within the yolk, no new formation of phosphatides (no formation of labeled phosphatides) takes place. This is shown by the fact that, if the labeled phosphate is administered after the egg has left the ovary, no active phosphatides are found in the yolk, as distinct from active inorganic phosphate which penetrates from the circulation into the egg during every phase of its formation.

The same fact is borne out by experiments *in vitro* in which eggs were placed for one day in a solution containing active phosphate. Of the activity found in the eggs, 99.4% was located in the shell, 0.4% in the white, and 0.2% in the yolk. The phosphatides extracted from the yolk were found to be inactive.

The egg enters the oviduct about 15 minutes after ovulation, passes

through the funnel in 18 minutes, spends 3 hours in traversing the magnum or albumin-secreting portion of the oviduct, 1 hour in the isthmus, and the remainder of the period (usually 20–24 hours) in the uterus. Thus, the egg spends about one day outside the ovary before being laid. When labeled phosphate was administered to a hen 5 hours before laying, the ovum was certainly no longer in the ovary. This egg (see Table XXV) did not contain active phosphatides, as was to be expected. Active non-phosphatide (mainly inorganic) phosphorus was found in the yolk, however, and the phosphorus of the white and the shell also showed very high activity.

TABLE XXV  
LABELED PHOSPHORUS CONTENT OF EGGS (85)

Time between administration of active P and egg laying	Per cent labeled P administered found in			
	Shell	Albumin	Total yolk	Yolk lecithin
5 hours	0.24	0.0015	0.0014	0.000
1.0 day	0.052	0.032	0.109	0.014
3.0 days	0.036	0.030	0.42	0.17
4.5 days	0.026	0.027	0.95	0.34
6.5 days	0.022	0.020	0.85	0.35

Shell deposition begins practically as soon as the egg reaches the uterus and presumably continues until oviposition. During the time the egg is in the uterus, approximately 5 g. of calcium carbonate, containing a small amount of phosphate (3–4 mg. phosphorus), is deposited in the shell membranes as the egg shell. This phosphate is secreted from a plasma containing highly active phosphate shortly after the administration of  $^{32}\text{P}$ , and the shell phosphate secreted shortly after administration of labeled phosphate is bound to be highly active. In the course of the next few days the activity of the plasma phosphate decreases and the shell of the eggs layed after a day or more is found to be less and less active, as seen in Table XXVI.

Lorenz, Perlman, and Chaikoff (118) showed that the amount of  $^{32}\text{P}$  deposited in phosphatides and other compounds in the yolk could be accounted for by an integral function of the two variables, yolk growth rate and  $^{32}\text{P}$  availability, during the corresponding period of new formation. These experiments also showed a marked dissimilarity in the deposition of phosphorus in shell and in albumin. Those shells that were being ac-

tively formed at the time of injection showed a high  $^{32}\text{P}$  content (up to 0.28% of the amount injected), whereas eggs that entered the uterus several hours later contained much smaller amounts of  $^{32}\text{P}$  in their shells (see Table XXV). Albumin protein is secreted while the egg is in the magnum and its deposition is completed about 22 hours before oviposition. Eggs laid during the 24- to 30-hour interval entered the magnum at a time when plasma radiophosphate was at its maximum. The  $^{32}\text{P}$  content in the albumin of these eggs did not exceed 0.05%, whereas eggs laid between 45 and 75 hours contained 2-4 times this amount. The delayed deposition of  $^{32}\text{P}$  in the albumin (see also 85) suggests that a synthetic process precedes the deposition of phosphorus-containing compounds. Egg albumin is known to contain slight amounts of phosphorus and it is not unlikely that the delay is due to the incorporation of phosphorus into this or other proteins before their deposition in the albumin.

Chaikoff and associates (46) determined what percentage of  $^{32}\text{P}$  administered was turned into phosphatide phosphorus in the laying and the nonlaying bird. The results are shown in Table XXVI.

TABLE XXVI  
PER CENT  $^{32}\text{P}$  ADMINISTERED AS PHOSPHATE FOUND IN PHOSPHATIDES OF LAYING  
AND NONLAYING BIRDS (46)

Organ	Laying bird		Nonlaying bird	
	6 hr.	12 hr.	6 hr.	12 hr.
Entire bird	3.62	4.55	3.25	4.57
Gastrointestinal tract	10	10	23	15
Muscle + bone + blood	32	36	27	35
Ovary + oviduct + yolks	11*	20	0.4	0.2
Liver	44	29	47	44

\* 10% in yolk, and only 1% in ovary and oviduct.

Table XXVI shows that nearly one-half the amount of active phosphatides of the bird is located in the liver, although the phosphatide content of the liver may be estimated to represent but 5% of that of the bird.

#### R. EFFECT OF DIETHYLSTILBESTROL ON TURNOVER OF PHOSPHATIDES

Flock and Bollman (52) investigated phosphatide turnover following administration of diethylstilbestrol to cocks. When radioactive sodium phosphate was given intraperitoneally to cocks, labeled phosphatides ap-

peared in the plasma in 2 hours and increased at a uniform rate for 12 hours. Six hours after the administration of  $^{32}\text{P}$  the specific activity of the phosphatide of plasma was similar in cocks which received diethylstilbestrol and in untreated birds. The concentration of phosphatides was, however, greater in plasma of treated than in plasma of untreated birds, and the total  $^{32}\text{P}$  content was correspondingly greater.

Calculations based on the  $^{32}\text{P}$  content of liver phosphatides and the amount and  $^{32}\text{P}$  content of phosphatides of plasma indicate that 1.51 mg. of phosphatide phosphorus entered each 100 ml. of plasma every hour in the untreated birds. Similar calculations showed an average of 5.0 mg. entering each hour in birds which had received diethylstilbestrol.

Calculations based on the disappearance of radioactive phosphatides from plasma of normal birds after intravenous injection of radioactive phosphatides into plasma indicate that 1.47 mg. of phosphatide phosphorus left each 100 ml. of plasma every hour. An average of 2.76 mg. left the plasma each hour in birds which had received diethylstilbestrol. Administration of diethylstilbestrol to birds appears to increase the rate of formation of phosphatides and also the rate at which they leave the plasma.

#### S. ORIGIN OF PLASMA PHOSPHATIDES

Strong additional evidence that plasma phosphatides, which in turn supply yolk phosphatides, are mainly derived from liver, is provided in investigations on formation of labeled phosphatides in the hepatectomized dog by Fishler *et al.* (50).

The observation that radiophosphatides are found in the kidney and small intestine of the hepatectomized dog leaves no doubt that the liver is not the only site of phosphatide formation in the animal body. The recoveries of phosphatide  $^{32}\text{P}$  per gram kidney phosphatide or per gram small intestine phosphatide in the liverless dog do not differ significantly from those found in the intact dog, the specific activities of kidney and intestinal mucosa phosphorus being, after the lapse of five hours, 65 and 41%, respectively, of that of liver phosphatide phosphorus.

Nevertheless, only negligible amounts of phosphatide  $^{32}\text{P}$  were recovered from the plasma of the hepatectomized dog as late as 6 hours after excision of the liver, as is seen in Table XXVIII. For sake of comparison the figures obtained for labeled phosphatide content of plasma and tissues of normal dogs are stated in Table XXVII.

Six hours after injection of labeled phosphate, the values for phosphatide  $^{32}\text{P}$  per gram tissue phosphatide were about 100 times greater in kidney

TABLE XXVII  
RECOVERY OF INTRAPERITONEALLY INJECTED  $^{32}\text{P}$  AS PHOSPHATIDE  $^{32}\text{P}$  IN PLASMA AND TISSUES OF NORMAL DOGS (50)

Dog weight, kg.	Time after injection, hr.	Organ weight, g.			Phosphatide content at end of experiment, mg./100 g. tissue					Recovery of phosphatide $^{32}\text{P}$ as per cent of injected $^{32}\text{P}$ per g. phosphatide				
		Liver	Kidney	Small intestine	Plasma	Liver	Kidney	Small intestine	Muscle	Plasma	Liver	Kidney	Small intestine	Muscle
17.1	6	326	44	216	264	2730	2260	1350	850	0.13	0.37	0.25	0.14	0.0084
9.6	6	308	41	226	462	3000	2340	1330	1080	0.15	0.32	0.19	0.15	0.0061
15.0	18	284	57	198	341	2280	2100	1410	1010	0.47	0.53	0.29	0.33	
10.5	18	213	37	278	345	3000	2160	1070	1220	0.55	0.52	0.40	0.40	
9.8	18	217	42	200	357	3120	2950	1040	1070	0.33	0.34	0.21	0.20	0.015
18.7	18	315	49	265	360	2780	2200	1160	1160	0.30	0.32	0.21	0.20	0.0092
9.5	36	292	52	276	219	2060	2100	1220		0.54	0.56	0.44	0.33	
10.2	36	246	50	266	272	2930	2620	1050	1280	0.42	0.41	0.29	0.38	0.012
7.0	98	228	27	201	271	2490	2430	1250	970	0.28	0.26	0.27	0.21	0.10
7.0	98	243	32	136	435	2580	2090	1350	1150	0.30	0.29	0.32	0.26	0.043
6.0	98	225	46	190	357	2740	2000	1180	1060	0.41	0.36	0.40	0.35	0.55

TABLE XXVIII

RECOVERY OF INTRAVENOUSLY INJECTED  $^{32}\text{P}$  AS PHOSPHATIDE  $^{32}\text{P}$  IN PLASMA  
AND TISSUES OF HEPATECTOMIZED DOG (50)

Dog weight, kg.	Time killed after <sup>32</sup> P injec- tion, hr.	Phosphatide content at end of experiment, mg./100 g. tissue			Recovery of phosphatide <sup>32</sup> P as per cent of injected <sup>32</sup> P			
					Plasma		Kidney	Small intestine
		Plasma	Kidney	Small intestine	Per ml. × 10 <sup>4</sup>	Per g. phosphatide × 10 <sup>4</sup>	Per g. phos- phatide	Per g. phos- phatide
Hepatectomy								
18.0	2	270	2380		0.1	0.3	0.25	
17.7	3	163	2030	1140	4	3	0.27	0.091
20.2	4	300			15	5		
20.0	4.5	210			19	9		
22.0	4.5	344	1820	1180	5	2	0.23	0.12
22.2	6	153	1470	1960	3	2	0.18	0.032
19.8	6	204	1870	1100	6	3	0.40	0.10
20.0	6	315	1860	2070	7	2	0.40	0.11
Sham operation								
19.5	6	232	2530	1120	170	72	0.43	0.12
19.5	6	282	2040	1300	318	110	0.26	0.12

than in plasma. If a transfer to plasma from kidney and small intestine occurs, it must be, in contradistinction to transfer to plasma from liver, a slow process; this result was arrived at also in experiments in which plasma containing labeled phosphatides was injected into the circulation (see page 152). This result strongly supports the conclusion that plasma phosphatides are derived mainly from liver.

#### T. TURNOVER OF VITELLIN

In an investigation carried out by Chargaff (37), the  $^{32}\text{P}$  content of the phosphorus of "free" lecithin and cephalin, of the "combined" phosphatides accompanying the vitellin fraction, and of the vitellin fraction were investigated. While about 50% of the phosphatides present in yolk, the "free" phosphatides, can be extracted with ether, the remainder, the "combined" phosphatides, are present as a constituent of the lipide-protein complex, lipovitellin, contained in hen egg yolk.

Phosphorus compounds isolated from yolks of eggs laid in the course of



eight days following intramuscular injection of radioactive sodium phosphate were examined individually. Each of the hens received two intramuscular injections. The second injections were given 18 hours after the first. The specific activities of the phosphorus extracted from the fractions, and hence the rate of formation of "free" lecithin and cephalin and

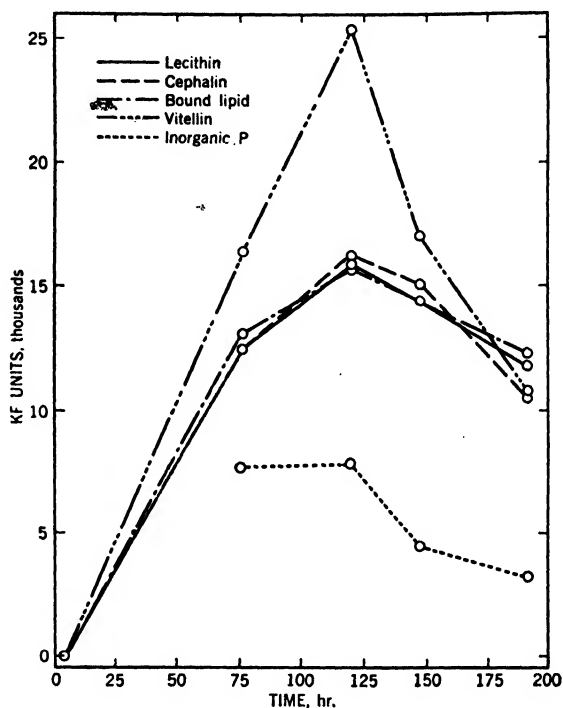


Fig. 14. Concentration of radioactive phosphorus in the yolk fraction (37).

of the "combined" phosphatides accompanying the vitellin fraction, were found to be equal. Vitellin phosphorus, however, exhibited a considerably higher specific activity in the first five to six days of the experiment than the phosphatide phosphorus. The maximum specific activity was obtained for all fractions after a lapse of six days (see Fig. 14).

The higher activity of vitellin can be explained by assuming that phosphatides were formed at a slower rate than vitellin, and, correspondingly.

that vitellin was formed in the first part of the experiment with the participation of more highly active phosphorus than were the phosphatides. If this explanation is correct, we should expect the vitellin phosphorus in the later phases of the experiment to be less active than the phosphatide phosphorus. This is actually found to be the case: after the lapse of eight days the vitellin phosphorus was found to be somewhat less active than the phosphatide phosphorus.

An additional reason for the higher activity of vitellin phosphorus in the first phase of the experiment may be sought in a greater dilution of the active phosphatides by the nonactive (old) phosphatides present in the organism. Substantial amounts of phosphatides are present in the organism, and part of these interchange with the plasma phosphatides. The latter will consequently be responsible for diluting the active phosphatides. On the other hand, vitellin is present only in small amounts in the hen, being readily detectable (148) only in the blood of laying hens.

The discovery of the presence of the hydroxyamino acid serine in brain phosphatides led Chargaff to suggest that a metabolic link existed between phosphoproteins and phosphatides, the mechanism possibly proceeding in the following steps: phosphorylation of the protein at the serine hydroxyl group, esterification of the phosphoprotein with diglycerides, decarboxylation of the latter to the corresponding ethanolamine-containing cephalins, etc. The above result, according to which the vitellin fraction contained more labeled phosphorus than the phosphatide fraction, can be interpreted as favoring this hypothesis.

#### U. ORIGIN OF PHOSPHATIDES IN CHICK EMBRYO

Only about two-thirds of the phosphatides in the egg are hydrolyzed during its incubation. Considering the large store of phosphatides in yolk (even shortly before the egg is hatched), we should expect the embryo to avail itself of this store when it needs phosphatides to build up its nervous system and other organs containing these substances. This point may be checked by introducing a small amount of labeled sodium phosphate, dissolved in 0.1 ml. of physiological sodium chloride solution, into the white of the egg before incubation, and determining whether, and to what extent, the phosphatides of the yolk and the embryo become labeled (88). If none of the phosphatides are labeled, we may conclude that the phosphatide molecules in the embryo are not newly synthesized from inorganic phosphate present there. If, on the other hand, the yolk phosphatide remains unlabeled while that of the embryo becomes radioactive, we may conclude

that the phosphatide molecules in the embryo have not come from the yolk but have been built up in the embryo with the participation of labeled inorganic phosphorus. As seen in Table XXIX, phosphatides extracted from the embryo invariably showed a high specific activity, while those from the yolk were barely active. The slight activity of yolk phosphatide, which increases with age of the embryo, is possibly due to an influx from the embryo into the yolk of a small amount of labeled phosphatides or of the enzymes responsible for resynthesis of phosphatides. The behavior of yolk phosphatides during incubation also illustrates the point discussed above, namely, that labeled phosphatides found in the yolk must have been deposited as such and that phosphatides once incorporated into the yolk cannot become labeled.

TABLE XXIX

RELATIVE SPECIFIC ACTIVITY OF PHOSPHATIDES EXTRACTED FROM EMBRYO  
AND RESIDUAL YOLK (88)

Time of incubation, days	Phosphatides extracted	Specific activity
6	Yolk	0.032
	Embryo	100
11	Yolk	0.10
	Embryo	100
18	Yolk	0.92
	Embryo	100

The specific activities of inorganic phosphorus, hexose monophosphate phosphorus, creatine phosphorus, phosphatide phosphorus, and residual phosphorus extracted from the embryo had the same value, showing that the inorganic phosphorus atom reaching the embryo has the same chance of entering a phosphatide or other molecule.

#### V. ORIGIN OF MILK PHOSPHATIDES

The origin of phosphatides of goat milk (15,16) was investigated along similar lines to studies of the origin of yolk phosphatides. Phosphatides extracted from milk a few hours after subcutaneous injection of labeled phosphate were found to be much more active than those present in the plasma. Thus, phosphatide molecules of the milk cannot have originated in the plasma, but must have been built up mainly in the mammary gland. The specific activity of the phosphatide phosphorus extracted from the

gland was, in fact, found to be higher than that of the corresponding products from the plasma and the milk, as is seen in Table XXX.

TABLE XXX

ACTIVITY OF PHOSPHATIDE PHOSPHORUS OF MILK AND ORGANS OF A GOAT (15)\*

Fraction	Relative specific activity per mg. phosphatide P
Milk.....	1
Plasma.....	0.02
Milk gland.....	1.4
Liver.....	1
Kidneys.....	1.2

\* 4.5 hr. after administration of labeled sodium phosphate.

Phosphatides are renewed in the milk gland at a still higher rate than in the liver. Investigation of the activity of different milk fractions by Aten (15) proved that no mixing occurred in the milk while stored in the udder. Moreover, it was found that, a few hours after the start of the experiment, the specific activity of the phosphorus of casein and of acid-soluble organic phosphorus compounds was but slightly lower than that of inorganic phosphate of the milk. This fact makes it seem very probable that these substances are formed in the milk gland from inorganic phosphate. The phosphorus atom is found to require 0.5 to 2.5 hours more to enter milk casein than to enter milk phosphate. The corresponding time for its entry milk phosphatides is over two days.

Kay and colleagues (64) have taken blood samples at different times after milking from the internal iliac artery and from the abdominal subcutaneous (mammary) vein of the cow and have determined phosphatide phosphorus, ester phosphorus, inorganic phosphorus, fatty acid, and other contents of these samples and of the corresponding plasma samples. No significant difference was found in content of phosphatide phosphorus an ester phosphorus, while inorganic phosphorus content of the plasma of the mammary vein was found to average 7%, and fatty acid content 2%, lower than the corresponding values for the iliac artery. From these findings it was concluded that the fat of cows' milk was derived in the main from nonphosphatide fatty acids of blood, i.e., probably from the fatty acids neutral fat. Phosphatides are thus not the blood precursors of milk

fat. Furthermore, it was concluded that phosphorus compounds of milk are mainly derived from inorganic phosphate of the blood plasma. These conclusions are in good accord with results arrived at by application of labeled phosphate to the study of precursors of phosphorus compounds in milk. Similar results were obtained in the study of the formation of labeled fatty acids and cholesterol in chick embryo (98).

#### W. TURNOVER OF PHOSPHATIDES IN CELL NUCLEI. EFFECT OF ROENTGEN RAYS ON PHOSPHATIDE TURNOVER

Cell nuclei contain appreciable amounts of phosphatides. About 12% of the dry nuclei of rat liver is composed of phosphatides; the corresponding value for dry liver tissue is 8. In hepatoma and adenocarcinoma of the rat, 8% and 7% are given for the phosphatide content of dry nuclei and dry tissue, respectively (183). Other authors state that phosphatide content of rat liver nuclei varies between 7.5 and 10.8% (42). Since nuclei make up but a minor part of wet tissue, the greater part of the phosphatides is located in cytoplasm.

A comparison of turnover rates of phosphatides in sarcoma (83) shows that no pronounced difference is found between rates of renewal of phosphatides in nuclei and in tissue, and correspondingly in cytoplasm. In nuclei of liver, however, rate of renewal of phosphatides clearly lags behind the rapid rate of turnover of these compounds in cytoplasm, as is seen in Table XXXI. To what extent failure to detect appreciable differences between turnover of phosphatides in nuclei and in tissue of sarcoma is due to a relatively rapid interchange between phosphatide molecules of cytoplasm and those of nuclei is not yet elucidated.

The effect of Roentgen rays on turnover rate of phosphatides present both in tissue and in nuclei was investigated as well. Two groups of twelve rats, after irradiation with 1000 r, are given labeled phosphate, while nonirradiated, control groups are treated in a similar way. After the lapse of two hours the animals are sacrificed and the sarcoma and livers are pooled separately. An aliquot is used in the determination of specific activities of inorganic and phosphatide phosphorus of the tissue, while from the bulk of the material cell nuclei are isolated by the method of Dounce (42). The specific activities of the corresponding phosphorus fractions of the nuclei are also determined, and furthermore the activity of the inorganic phosphorus of the pooled blood plasma is measured. As seen in Tables XXXI and XXXII, the rate of turnover of phosphatides in liver

nuclei is markedly diminished by the action of Roentgen rays; that of the cytoplasm is also diminished though to a lesser extent.

TABLE XXXI

EFFECT OF ROENTGEN RAYS ON TURNOVER OF PHOSPHATIDES OF CELL NUCLEI AND TOTAL TISSUE IN JENSEN SARCOMA AND RAT LIVER (83)

Groups of 12 rats of 150-g. av. wt.	Activity of 1 mg. phosphatide P as per cent of activity of 1 mg. inorganic tissue P			
	Sarcoma		Liver	
	Nuclei	Tissue	Nuclei	Tissue
Controls	2.86	3.75	10.34	20.44
Controls	2.38	2.37	8.15	11.25
Controls	2.75	2.67	11.33	14.25
Irradiated	2.56	2.32	3.29	12.30
Irradiated	1.02	1.52	4.08	8.91
Irradiated	1.44	1.41	4.83	8.20
Per cent decrease due to irradiation	37	38	59	36

TABLE XXXII

EFFECT OF ROENTGEN RAYS ON ACTIVITY OF PHOSPHATIDE PHOSPHORUS OF NUCLEI AND TISSUE OF RAT LIVER AND SARCOMA

Animals	Activity of 1 mg. phosphatide P of rat sarcoma and liver as per cent of activity of 1 mg. inorganic P of blood plasma			
	Sarcoma		Liver	
	Nuclei	Tissue	Nuclei	Tissue
Controls	3.14	4.07	8.72	17.80
Controls	2.73	2.71	11.23	15.68
Controls	3.30	3.20	9.26	11.62
Irradiated	2.23	2.04	3.19	11.35
Irradiated	0.99	1.48	2.93	6.36
Irradiated	1.28	1.25	3.88	6.60
Per cent decrease due to irradiation	50	52	66	31

## X. APPLICATION OF INDICATORS OTHER THAN RADIOPHOSPHORUS IN STUDY OF PHOSPHATIDE METABOLISM

### 1. Incorporation of Deuterium-Containing Fatty Acids

Cavanagh and Raper (31) fed rats labeled fat which had been prepared by the partial saturation of unboiled linseed oils with deuterium. Deute-

rium comprised 4–5% of the hydrogen atoms of the fat. The deuterium contents of phosphatides extracted from various tissues of the rats were then determined at various times. The presence of deuterium-containing phosphatides in liver and other organs a few hours after feeding the linseed oil is shown in Table XXXIII.

TABLE XXXIII

DEUTERIUM CONTENT OF PHOSPHATIDE FRACTIONS AFTER FEEDING FAT CONTAINING 4–5 ATOM PER CENT DEUTERIUM (31)

Organ	Atoms per cent D after		
	6 hours	10 hours	24 hours
Plasma	0.12	0.47	..
Liver	0.47	0.52	0.42
Kidney	0.16	0.14	0.17
Brain	0.004	0.08	0.03

At all intervals the highest values for deuterium content were observed in liver phosphatides. The smallest incorporation of deuterium was observed in brain phosphatides, and thus the result obtained was similar to results of the incorporation of  $^{32}\text{P}$  into phosphatides of different organs. The observation that plasma phosphatides have lower deuterium values than liver at the end of six hours was interpreted as suggesting incorporation of deuterated fatty acids into liver phosphatide. A similar conclusion was drawn concerning incorporation of phosphate into the phosphatide molecule (see page 156).

After keeping mice on a stock diet containing 20% deuterated linseed oil for three days, the liver phosphatide fatty acids were found, by Barrett and his associates (21), to contain nearly three times as much deuterium as body phosphatides.

## 2. Change in Degree of Unsaturation of Fatty Acids

Since phosphatides contain both saturated and unsaturated fatty acids, change in composition of the fatty acids of the organ phosphatides after ingestion of cod liver oil, for example, can be utilized in securing information regarding rate of phosphatide renewal in the organ in question. A change in iodine number of phosphatides extracted from the livers of dogs (96) and cats (165) after ingestion of cod liver oil, and disappearance of the

changes within 24 hours and 2-3 days, respectively, have been observed in early experiments.

### *3. Incorporation of Iodinated Fatty Acids*

Iodinated fatty acids, whether injected intravenously or given by mouth, enter the phosphatides of liver, blood (12), and milk (18), for example.

### *4. Incorporation of Elaidic Acid*

This method has been used repeatedly by Sinclair and others in the investigation of rate of renewal of phosphatides. Rate of entry of elaidic acid into and disappearance from phosphatides was found to be rapid in liver and intestinal mucosa and comparatively slow in muscle. The process was found to be essentially complete in liver within a day, but in muscle only after the period of many days (166, 167).

Administered elaidic acid was found by Sinclair (166) to appear early in plasma phosphatides of the cat. A rapid replacement of phosphatide fatty acids of plasma is indicated by the observation that, eight hours after the feeding of elaidic acid, about 20% of the plasma phosphatide fatty acids consisted of this acid. Maximum incorporation after feeding occurs in less than one day in small intestine and in slightly more than one day in liver. In kidney and muscles, one-half of the maximum incorporation occurs in about three days. The rate of entry of elaidic acid into phosphatides of carcinosarcoma 256 is slower than into liver phosphatides but faster than entry into muscle phosphatide (73), a behavior also shown by labeled phosphate.

The fact that phosphatide fatty acids of different tissues can be replaced only partly by elaidic acid alone restricts the applicability of elaidic acid as indicator. Not more than 30% of phosphatide fatty acids of liver and of small intestine can be replaced by elaidic acid, and not more than 7% of those of brain and still less in testes can thus be replaced. Furthermore, in order to obtain quantitative results from experiments carried out with elaidic acid as indicator, we should know, beside the elaidic acid content of the phosphatides, the elaidic acid content of the fatty acid mixture available for phosphatide synthesis in cells of the organ. Lack of knowledge of the concentration of the tracer in the precursor of the compound whose rate of formation we wish to determine is the greatest obstacle in the application of isotopic and nonisotopic indicators to determination of turnover rate.



### *5. Incorporation of Fatty Acids with a Characteristic Absorption Spectrum*

Linoleic acid of corn oil is converted by prolonged saponification to a fatty acid containing conjugated double bonds with high spectral absorption. Such fatty acids can be identified by their characteristic absorption spectra (128). Conjugated fatty acids, when administered by mouth, are incorporated in phosphatides of intestinal mucosa. About 6% of the phosphatide fatty acids are found to be replaced by labeled fatty acids after the lapse of one hour and a maximum of 15% after eight hours.

Barnes and his associates (128) found that the rates of entry of conjugated fatty acids into phosphatides of intestinal mucosa and liver were not impaired in the adrenalectomized rat. In fat-deficient rats, however, a decrease in incorporation of the labeled fatty acids took place. Introduction of the tagged fatty acids into phosphatides of intestinal mucosa can also be demonstrated in experiments with tissue slices.

### *6. Incorporation of Heavy Nitrogen ( $^{15}\text{N}$ ) in Phosphatides*

Three days after choline, containing  $^{15}\text{N}$ , had been administered, 21% or more of the choline present as phosphatide in the whole animal was found to be replaced by isotopic dietary choline. The corresponding replacement figure for labeled ethanolamine was 28% or more. The choline content of liver is renewed at the highest rate. Renewal of the choline of the gastrointestinal tract is slower than that in liver, slowest replacement takes place in the brain (170).

### *7. Incorporation into Phosphatides of Analogs of Choline in Which Arsenic Replaces Nitrogen*

Arsenic can be detected in the lecithin fraction isolated from liver and brain of rats kept for 21 days on a diet containing arsenocholine chloride (180).

We thus complete the review of different methods in which isotopic and nonisotopic indicators are so far applied in investigating the rate of renewal of the phosphatide molecules. The application of these methods demonstrates that renewals of the phosphate group, the fatty acid, and the nitrogen base group of phosphatide molecules take place. Replacement in liver is most pronounced; that in brain most sluggish. We cannot, however, yet state whether the rates of renewal of these three components differ and, if so, to what extent. Nor is it possible yet to determine to what degree phosphorylation processes are involved in fat absorption. A com-

parison of phosphatide molecules turned over in intestinal mucosa with amount of fat absorbed may be expected to bring this problem nearer its solution.

#### IV. Turnover of Nucleic Acids

##### A. TURNOVER OF DESOXYRIBONUCLEIC ACID

Desoxyribonucleic acid is wholly or mainly confined to cell nuclei. Since the desoxyribonucleic acid content of cell nuclei increases and decreases in the various stages of mitosis, we can expect an appreciable turnover of nucleic acid to take place in growing tissue, and in such organs as well which secrete products containing desoxyribonucleic acid. To the latter belong, among others, thymus, spleen, and bone marrow.

Liver and kidney of mature animals show, as is to be expected, a very low rate of renewal of their desoxyribonucleic acid content. The percentage ratio of activity of 1 mg. desoxyribonucleic acid phosphorus and of 1 mg. inorganic phosphorus in experiments taking two hours is found to be about 0.1 mg. both in liver (3,71,90) and in kidneys (3,90), though in the liver in some cases higher values are obtained.

The corresponding ratio is very much larger in the case of spleen and intestinal mucosa, as seen in Table XXXIV and Figure 15.

The highest rate of renewal is found, as shown by Andreassen and Ottesen (10), in bone marrow and thymus gland of the rat.

The data in Figure 15 are obtained by comparing specific activity of desoxyribonucleic acid P at the end of the experiment (94 hr.) with the average value prevailing throughout the experiment of specific activity of inorganic phosphorus of the organ. It is thus assumed that the inorganic phosphate, or a phosphorus-containing precursor which attains relatively rapid equilibrium with inorganic phosphate, is incorporated in the newly formed desoxyribonucleic acid molecules.

Calculation of the above figures was made without regard to repeated renewal of some of the molecules during the experiment. The

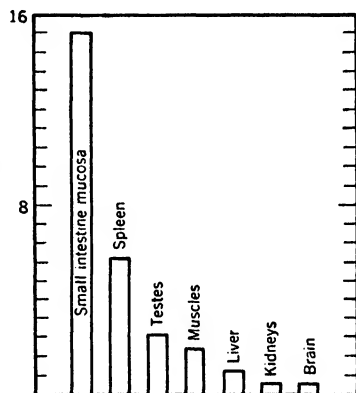


Fig. 15. Daily percentage rate of formation of labeled desoxyribonucleic acid in the organs of the rat.

values stated for daily percentage renewal in intestinal mucosa and, to a still more restricted extent, in some other organs, may be correspondingly somewhat low.

In Table XXXIV the percentage ratios of specific activities of desoxyribonucleic acid phosphorus and of inorganic phosphate, both at the end

TABLE XXXIV

SPECIFIC ACTIVITIES OF DESOXYRIBONUCLEIC ACID PHOSPHORUS AND INORGANIC PHOSPHORUS TWO HOURS AFTER LABELED PHOSPHATE ADMINISTRATION TO MATURE RAT (90)

Organ	Percentage ratio of specific activities of desoxyribonucleic acid P and inorganic P
Liver.....	0.14
Spleen.....	2.50
Kidneys.....	0.16
Intestinal mucosa.....	4.80

of a two-hour experiment, are recorded. The figures represent the average of numerous values obtained. The end value of specific activity of liver inorganic phosphate hardly differs from the mean value in experiments taking two hours. For spleen the end value is about 25% higher than the mean value (3,11).

It can be assumed that much of the turnover of desoxyribonucleic acid takes place in dividing or secreting cells and it is quite possible that such cells are more permeable to phosphate than the average cell of the tissue in question. Such an enhanced permeability would not much influence the renewal figures obtained for liver desoxyribonucleic acid, as within short time the specific activity of inorganic phosphorus of liver reaches the value in plasma. In the case of organs which show a restricted cell permeability, as for example in brain, testes, or muscle, in experiments taking two hours tissue inorganic phosphorus has, however, an appreciably lower specific activity than plasma inorganic phosphorus. In the case of these organs it is possible that desoxyribonucleic acid is synthesized from inorganic phosphorus having a higher specific activity than the inorganic phosphorus extracted from tissue; thus the renewal values arrived at are too high. The correct renewal figures, however, cannot be lower than the figures obtained by comparing the specific activity of desoxyribonucleic acid phosphorus of the organ with that of the inorganic phosphorus of plasma. The latter figures are given in Table XXXV. It is advisable in these, and also in

TABLE XXXV  
SPECIFIC ACTIVITIES OF DESOXYRIBONUCLEIC ACID OF SOME ORGANS AND THAT  
OF INORGANIC PHOSPHORUS PLASMA (3)

Organ	Percentage ratio of specific activities of 1 mg. desoxyribonucleic acid P and of 1 mg. plasma inorganic P
Kidney.....	0.15
Liver.....	0.20
Spleen.....	1.79
Intestinal mucosa.....	2.1

other turnover experiments (86), to determine both the ratio of the specific activity of the phosphorus of the organic compound in question to the specific activity of tissue inorganic phosphorus and the ratio of the former to the specific activity of plasma inorganic phosphorus. If the formation of the organic compound is preceded by an organic precursor, the ratio of the specific activities of the phosphorus of the final product and the precursor should be determined as well.

Experiments in which the specific activity of desoxyribonucleic acid of liver was investigated several days after administration of labeled phosphate (29) are discussed on page 180. In surviving Jensen sarcoma tissue slices shaken with a Ringer solution containing labeled phosphate at 37°C., the formation of minute amounts of labeled desoxyribonucleic acid was found to take place (6).

Andreassen and Ottesen (11) determined the percentage ratio of the injected amount of  $^{32}\text{P}$  in 1 mg. desoxyribonucleic acid and 1 mg. inorganic plasma phosphate of lymphoid organs in experiments taking 3 hours in infant, young mature, and old rats. Their results (average of 3 experiments) in Table XXXVI indicate a decrease with age of the animal in rate of formation of labeled desoxyribonucleic acid in thymus, lymph nodes, and spleen, the decrease being partly due to a decreasing rate of penetration of the radioactive tracer into the tissues. This rate of penetration for lymph nodes and spleen was found to be appreciably lower than for thymus.

In organs of growing rats, beside renewal of nucleic acid molecules, an appreciable additional formation of nucleic acid takes place, since the nucleic acid content of the organs increases with increasing weight. That the percentage ratio of labeled, and thus newly formed, nucleic acid molecules is much larger in 3.5-day old than in mature rats is seen by comparing the figures of Tables XXXIV and XXXVII (47).

TABLE XXXVI

DESOXYRIBONUCLEIC ACID TURNOVER IN LYMPHOID ORGANS OF RATS OF DIFFERENT AGE IN EXPERIMENTS TAKING THREE HOURS (11)

Organ	Percentage ratio of specific activity of nucleic acid P of organ and inorganic P of plasma		
	30-day old rats	110-day old rats	143-day old rats
Thymus	7.6	5.3	5.6
Lymph nodes of intestines	6.1	1.5	1.0
Lymph nodes of skin	2.9	1.4	1.1
Spleen	2.4	1.6	0.8
Bone marrow	...	10	13

TABLE XXXVII

SPECIFIC ACTIVITIES OF NUCLEIC ACID P AND INORGANIC P TWO HOURS AFTER ADMINISTRATION OF LABELED PHOSPHATE TO 3.5-DAY OLD RATS (47)

Organ	Percentage ratio of specific activities of desoxyribonucleic acid P and free P
Liver.....	1.96
Spleen.....	9.76

The percentage increase in total desoxyribonucleic acid content of liver of the 3.5-day old rat amounts, in the course of two hours, to 0.9%. About half of the newly formed (labeled) desoxyribonucleic acid molecules present are due to increase in nucleic acid content of liver, while the other half are due to renewal of old molecules. A similar result was obtained in the investigation of formation of labeled nucleic acid in Jensen sarcoma. While in the course of two hours the increment in nucleic acid content of the sarcoma is found to average 1.5%, labeled nucleic acid molecules formed during this time amount to about twice this value (1,47).

It is of interest to compare rate of renewal of the phosphorus of desoxyribonucleic acid of liver with rate of renewal of liver proteins. Shemin and Rittenberg (163) found, by using  $^{15}\text{N}$  as an indicator, that, in seven days, half the liver protein was replaced by nitrogen from the diet and other proteins. Thus the rate of renewal of desoxyribonucleic acid of liver is only about 20% the rate of renewal of liver proteins.

After administration of labeled phosphate to mice, the  $^{32}\text{P}$  content of nucleoproteins (containing both desoxyribose and ribose compounds) of

normal livers and of livers in which cancer was produced by feeding azo dyes was compared. Tumorous livers, in which a rapid formation of new cells takes place, were found to exhibit an increased uptake compared with the controls (109). In sarcoma slices incubated four hours at 37°C. in plasma containing labeled phosphate, about 0.1% of the desoxyribonucleic acid molecules are found to have been renewed (6). In red corpuscles of the hen no renewal of appreciable amounts of desoxyribonucleic acid present takes place (12).

In view of the low activity of desoxyribonucleic acid it is of great importance to purify very carefully the sample from other, more active phosphorus compounds present. Such a purification can be obtained by repeated solution in an alkaline medium and precipitation with a solution of hydrogen chloride in methanol (9).

In earlier investigations of turnover of nucleic acid, acid-soluble and phosphatide components of tissue were extracted with trichloroacetic acid and with ether-alcohol, and the activity of the residual part was determined. Such residues contain, beside thymonucleic acid, ribonucleic acid and possibly also phosphoproteins. Since the rate of renewal of ribonucleic acid is much larger than that of thymonucleic acid, no conclusion about the value for thymonucleic acid can be drawn from these experiments.

#### B. EFFECT OF ROENTGEN RAYS ON TURNOVER OF DESOXYRIBONUCLEIC ACID

It is mainly the effect of radiation on the cell nucleus that blocks cellular division. This was most directly demonstrated in the following experiment (143). Agar cultures containing the alga *Zygnema* were irradiated with  $\alpha$ -rays emitted by polonium. In one series of experiments, both cytoplasm and nucleus were irradiated, while, in another series, the range of the  $\alpha$ -particles was reduced to such a value as to expose only the cytoplasm to the effect of irradiation. In the latter case, the dose necessary to suppress cellular division was found to be several hundred times larger than in the former (cf. 121).

In view of the importance of desoxyribonucleic acid in cell division, the effect of roentgen rays on the turnover of desoxyribonucleic acid phosphorus was investigated (1,3,4,47). Roentgen radiation (100 r or more) was found to decrease turnover rate of desoxyribonucleic acid, in both Jensen sarcoma and normal organs. As seen in Table XXXVIII the amount of newly formed desoxyribonucleic acid molecules, found to be 2-3% in the course of two hours in nonirradiated, growing sarcoma, is reduced by irradiation to one-half to one-third of its normal value. These results are ob-

tained in experiments in which the labeled phosphate is administered after irradiation.

TABLE XXXVIII  
EFFECT OF IRRADIATION OF JENSEN RAT SARCOMA ON FORMATION  
OF DESOXYRIBONUCLEIC ACID (1)

Dosage, r	Time between irradiation and injection	Time between injection and killing of rat, hr.	Ratio of newly formed nucleic acid in controls and in irradiated sarcoma
750-1500	Several minutes	0.5	3.2
335-1500	" "	1	2.4
450-1500	" "	2	2.2
1500	" "	4-6	2.8
1230-1500	3 to 7 days	2	1.7

In experiments with rats with two inoculated sarcomata, one sarcoma was irradiated while the other was effectively shielded. A reduction of labeled nucleic acid formation was found to take place in both; the reduction was smaller in the shielded than in the irradiated sarcoma (4, see also 5).

Roentgen radiation blocks the turnover of desoxyribonucleic acid molecules not only in growing tissue, but also in normal organs of adult animals (3), as is seen in Table XXXIX.

TABLE XXXIX  
RATIO OF NEWLY FORMED DESOXYRIBONUCLEIC ACID MOLECULES PRESENT  
BEFORE AND AFTER IRRADIATION IN THE ORGANS OF ADULT RATS (4)\*

Organ	Ratio of newly formed nucleic acid in the organs of controls and of irradiated rats
Liver.....	3.3
Spleen.....	2.4
Intestinal mucosa.....	2.3

\* Dose applied 1480-3000 r. Rat irradiated *in toto*. <sup>32</sup>P injected after the irradiation. Rat killed two hours after administration of the <sup>32</sup>P.

The effect of irradiation on formation of nucleic acid molecules in organs of normal adult rats is thus similar in magnitude to the effect of roentgen rays on the growing Jensen sarcoma; listed in Table XXXVIII.

Roentgen rays were found to block formation of nucleic acid in organs of rapidly growing rats also. The percentage inhibition of the desoxyribonucleic acid turnover was found to be similar to that in normal organs of adult animals and in Jensen sarcoma.

The ratio of newly formed desoxyribonucleic acid in organs of controls and in irradiated rats was found to be 2.3 in experiments taking two hours, in which the 3- to 4-day old rats were irradiated with 2000–2250 r previous to administration of labeled phosphate.

Irradiation throughout the experiment was found to reduce formation of labeled desoxyribonucleic acid to a still greater extent than that stated above (2). In these experiments, however, when the rats were fixed to a table and irradiated after injection of labeled phosphate, the low  $^{32}\text{P}$  content of desoxyribonucleic acid was to a large extent due to disturbed circulation, which prevented the isotopic tracer from reaching the sarcoma cells.

### C. RATE OF RENEWAL OF RIBONUCLEIC ACID

In contradistinction to desoxyribonucleic acid—the main constituent of cell nuclei—ribonucleic acid, which according to Brachet (28) and Caspersson (30) is generally found in cytoplasm, is renewed at a fairly rapid rate in the organs. Hammarsten and Hevesy (71) found, for the

TABLE XL

RATE OF RENEWAL OF RIBONUCLEIC AND DESOXYRIBONUCLEIC ACID IN ORGANS OF RAT  
TWO HOURS AFTER ADMINISTRATION OF LABELED PHOSPHATE (71)

Organ	Nucleic acid	Percentage ratio of specific activity of nucleic acid P to that of inorganic P of organ	Ratio of specific activity of ribonucleic acid P to that of desoxyribonucleic acid P
Liver	Ribose	3.45	33
	Desoxyribose	0.105	
Spleen	Ribose	6.6	3
	Desoxyribose	2.2	
Intestine	Ribose	6.1	2
	Desoxyribose	2.8	

percentage ratio of the specific activities of desoxyribonucleic acid phosphorus and inorganic phosphorus, and, for that of ribonucleic acid phosphorus and inorganic phosphorus, two hours after subcutaneous administration of labeled sodium phosphate, the values recorded in Table XL.



As Hammarsten finds, 70% of the nucleic acids of liver (a similar value of 75% was reported by Davidson and Waymouth (40), and 44% of those of spleen are of the ribose type. In liver, in experiments taking two hours, 99% of the  $^{32}\text{P}$  content of the nucleic acids turns out to be present in the ribose compound, the corresponding figure in the spleen being 70.

Brues and his associates (29) determined the specific activity of both the ribonucleic acid and the desoxyribonucleic acid of resting and regenerating liver and also of hepatoma. In these experiments the labeled phosphate was administered several days before the rats were killed.

Both nucleoproteins are precipitated with calcium chloride and dextroxyribonucleoprotein was separated by treatment with sodium chloride. Ribonucleic acid was purified by precipitation of the barium salt and subsequent precipitation of the free acid in glacial acetic acid.

The results obtained, seen in Table XLI, show that the rate of renewal of ribonucleic acid is higher than that of desoxyribonucleic acid and that turnover in regenerating liver and hepatoma is more rapid than in resting liver. That the ratio of specific activities of ribonucleic and desoxyribonucleic acid is found, in these experiments, to be appreciably smaller than in the experiments of Hammarsten and Hevesy, may be due, at least in part, to the much longer duration of the former experiments.

TABLE XLI  
SPECIFIC ACTIVITIES OF NUCLEIC ACID PHOSPHORUS (29)

Organ	Time after injection, days	Nucleic acid, per cent of specific activity of inorganic P		Ratio
		Ribose	Desoxyribose	
Resting liver	3	54.9	10.6	5.2
	8	123	20.8	5.9
Regenerating liver	3	230	180	1.3
	13	314	576	0.5
Hepatoma	3	171	64	2.7

Kohman and Rusch (109) administered labeled phosphate to rats and mice and determined the  $^{32}\text{P}$  content of nucleoproteins (containing both desoxyribose and ribose compounds) of normal liver and of liver in which cancer was produced by feeding azo dyes. The tumorous liver, in which a rapid formation of new cells takes place, was found to have a 45% increase in uptake of  $^{32}\text{P}$ , compared with the normal liver.

#### D. SPECIFIC ACTIVITY OF NUCLEIC ACID PHOSPHORUS OF THE WHOLE RAT

Hammarsten (71) determined the specific activity of both total desoxyribonucleic acid phosphorus and total ribonucleic acid phosphorus extracted from a rat weighing 194 g. The activity of labeled sodium phosphate administered amounted to 8.1 microcuries per 100 g. animal weight. The time of the experiment was two hours. The results of this experiment are shown in Table XLII. As shown in the table, the specific activity of

TABLE XLII

SPECIFIC ACTIVITIES OF NUCLEIC ACID PHOSPHORUS OF WHOLE RAT, LIVER, SPLEEN, AND INTESTINAL MUCOSA (71)

Sample	Specific activity (whole rat ribonucleic P = 100)		
	Ribonucleic	Desoxyribonucleic	Inorganic P
Total rat	100	60	
Liver	164	4.4	5100
Spleen	292	63	2850
Intestine	112	63	2770

the average nucleic acid phosphorus of the rat is almost identical with the value for ribo- and desoxyribonucleic acids, respectively, extracted from the intestine.

Interpretation of the significance of specific gravity figures obtained for the whole rat poses some difficulties, since the specific activity of the inorganic phosphorus utilized in the formation of labeled nucleic acid molecules is unknown. If the specific activity of the inorganic phosphorus utilized in building up the average body nucleic acid were similar to the specific activity of inorganic liver phosphorus, the percentage rate of renewal of body ribo- and desoxyribonucleic acids would be 2.0 and 1.2, respectively. If the specific activity of inorganic phosphorus utilized in building up the average nucleic acid of the organism were similar to the specific activity of inorganic intestinal phosphorus, larger values, *i.e.*, 3.6 and 2.2, respectively, would be obtained.

It is improbable that inorganic phosphate of such great activity is utilized in synthesis of nucleic acid as is found in a two-hour experiment in liver. Liver and kidneys have a privileged position in rate of uptake of phosphate. The amount of nucleic acid present in liver and kidneys makes up, furthermore, only a small percentage of total nucleic acid content of the organism. It is much more probable that inorganic phosphorus of similar

specific activity as found in the intestine is used in building up the labeled nucleic acid molecules. In fact, the amount of nucleic acid present in the mucosa of the digestive tract makes up a large percentage of body nucleic acid. While body nucleic acid contains slightly radioactive fractions, namely, those originating from liver, kidneys, and brain, and those fractions of restricted radioactivity originating from muscle, it contains also fractions of higher activity than found in the intestinal mucosa, namely, those originating from bone marrow and thymus. The lymphocytes secreted into the organism can also be expected to contain markedly active nucleic acid. This makes it understandable that the rate of renewal of average body nucleic acid corresponds to about the rate of renewal of intestinal nucleic acid and is thus considerable for both types of nucleic acid, in contradistinction to the rate of renewal found in liver, for example, which is very low for desoxyribonucleic acid and appreciably higher in the case of ribonucleic acid.

Andreassen and Ottesen (11) attempted to estimate lymphocyte production in different lymphoid organs from the rate of turnover of desoxyribonucleic acid in these organs. They concluded that the thymus must be the most important lymphocytopoietic organ except in old age.

## V. Phosphorus Turnover in Cell Nuclei

Marshak (122,123) has isolated nuclei from liver and lymphoma tissue of rats weighing 150 g., and has shown that most  $^{32}\text{P}$  taken up by the nuclei is present in the residue obtained after extraction with trichloroacetic acid and an ether-alcohol mixture. The phosphorus compound of the residue

TABLE XLIII  
DISTRIBUTION OF  $^{32}\text{P}$  IN CELL NUCLEI (123)

Organ	Time after injection	$^{32}\text{P}$ distribution in nuclei, %				
		Water soluble	Acid soluble	Phosphatide	Residue	Total
Liver	1 hr.		6.0		67.2	94.6
	1 day	15.2	3.3	6.1	68.3	92.9
	3 days	26.1	0.8	4.7	66.0	97.6
	5 days	2.7	1.9	8.6	66.9	80.1
	7 days	12.6	7.7	7.7	71.6	99.6
Lymphoma	1 day	2.5	0.6	0.6	94.8	98.5
	2 days	1.5	0.7	2.3	95.4	99.9
	3 days	3.6	2.5	0.8	94.5	101.4
	5 days	2.1	3.4	1.4	91.6	100.1
	7 days	8.4	26.4	0.8	88.1	93.9
				1.4	70.4	106.6

is presumably exclusively nucleoprotein. The distribution of  $^{32}\text{P}$  in the nuclei is seen in Table XLIII.

Chemical analysis of the phosphorus of the liver nuclei shows that the  $^{31}\text{P}$  content of the residual fraction makes up 81% of the total phosphorus

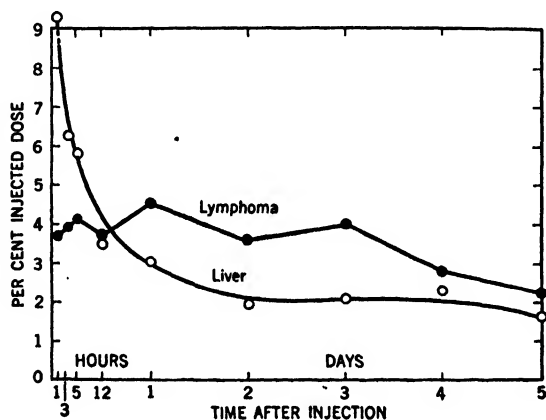


Fig. 16A.  $^{32}\text{P}$  uptake by tissues (123).

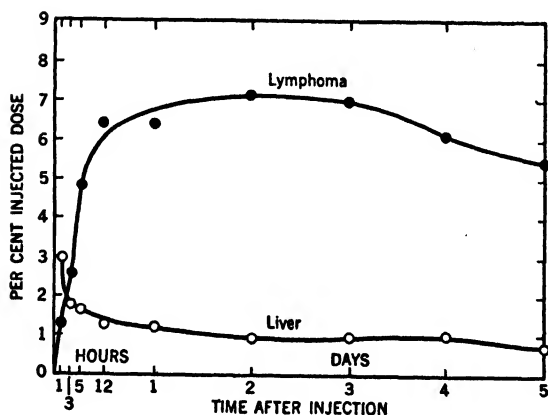


Fig. 16B.  $^{32}\text{P}$  uptake by nuclei (123).

content, the corresponding value for phosphatide, acid-soluble, and water-soluble fraction being 7, 6, and 7%, respectively. The residues of the tumor nuclei contain 90–95% of the total nuclear activity at all times

until the seventh day after injection of the labeled phosphate. Similar results were obtained in the investigation of  $^{32}\text{P}$  uptake by nuclei of sarcoma 180.

That  $^{32}\text{P}$  accumulates in nuclei of rapidly growing tumor to a much greater extent than in fully grown liver is demonstrated by Figures 16 and 17. This difference in accumulation of  $^{32}\text{P}$  in nuclei of liver and of lymphoma is to be expected in view of the facts that most of the phosphorus

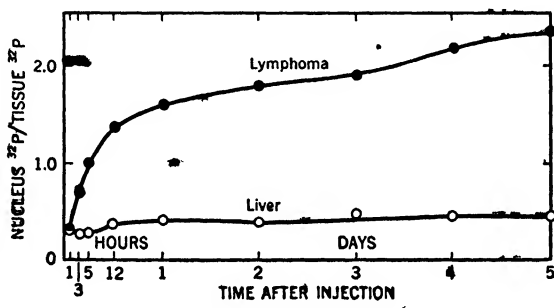


Fig. 17. Nuclear activity as a fraction of liver and lymphoma tissue activity (123).

of nuclei is present as deoxyribonucleic acid phosphorus, and that turnover of this compound is exceedingly low in nuclei of fully grown liver (see page 175) but is appreciable in nuclei of rapidly growing tumor tissue or even in nuclei of rapidly growing normal tissue.

That the rapid accumulation of  $^{32}\text{P}$  by nuclei may be attributed to mitotic activity is suggested by the following experiment carried out by Marshak (123).

The median and left lateral lobes of the livers of three 150-g. rats were removed, and labeled phosphate was injected by way of the femoral vein 36 hours later. Normal rats and rats bearing bilateral carcinoma 256 implants were injected at the same time. Two days later the livers were cleared of blood by perfusion with saline, and the nuclei were isolated from the livers and tumors. For the normal liver the ratio of activity per milliliter of nuclei to activity per gram of tissue was 0.345, while for regenerating liver and tumor the ratios were 1.02 and 1.08, respectively.

When  $^{32}\text{P}$  was injected four days after partial hepatectomy and the nuclei were isolated three days later, the ratios for normal and for hepatectomized animals were 0.28 and 0.32. At this time very few nuclei were found in mitosis, while thirty-six hours after partial hepatectomy 3.7% were in the anaphase or metaphase stages and many more were in the prophase stage.

The author (78) investigated the uptake of  $^{32}\text{P}$  by the tissue and nuclei of liver and Jensen sarcoma of the rat. In these investigations the method of separation of nuclei introduced by Dounce (42) was applied. The ratio of specific activity of liver tissue phosphorus and liver nucleus phosphorus found in the different experiments is shown in Table XLIV, showing that

TABLE XLIV  
RATIO OF SPECIFIC ACTIVITY OF TOTAL TISSUE PHOSPHORUS AND TOTAL NUCLEUS  
PHOSPHORUS IN LIVER OF RATS

Time after administration of $^{32}\text{P}$ , hr.	Ratio of specific activity of total tissue P to that of total nucleus P
1 (Marshak)	3.7
2 (Hevesy)	7.2 (Jensen sarcoma 5.2)
3 (Marshak)	2.5
5 (Marshak)	2.5

TABLE XLV  
RATE OF RENEWAL OF TOTAL, PHOSPHATIDE, AND RESIDUAL PHOSPHORUS IN THE TISSUE  
AND NUCLEI OF LIVER AND JENSEN SARCOMA OF THE RAT (82)\*

Fraction	Ratio of specific activity of fraction P to inorganic P of the organ
Liver	
Total tissue P	27.80
Total nucleus P	3.86
Tissue phosphatide P	15.34
Nucleus phosphatide P	9.95
Nucleus residual P	3.21
Sarcoma	
Total tissue P	18.80
Total nucleus P	3.59
Phosphatide P	2.83
Nucleus phosphatide P	2.66
Nucleus residual P	3.16

\* Time of experiment = 2 hours. Mean values for 36 rats.

an appreciable part of the labeled phosphate finds its way from the cytoplasm into the nuclei. Table XLV contains data on specific activities of total phosphorus and phosphatide phosphorus of the tissue and nuclei, and also on residual phosphorus (phosphorus remaining after treatment with trichloroacetic acid, ether-alcohol and chloroform) of nuclei. As the separation of nuclei takes place in acid solution, part of the acid-soluble phos-

phorus present in the nuclei is removed during their isolation and thus the value obtained for total  $^{32}\text{P}$  content of nuclei depends upon the procedure employed.

## VI. Phosphorus Turnover in Leukemic Tissue

In their studies on phosphorus metabolism of leukemic tissue Tuttle, Erf, and Lawrence compared the distribution of labeled phosphorus in the

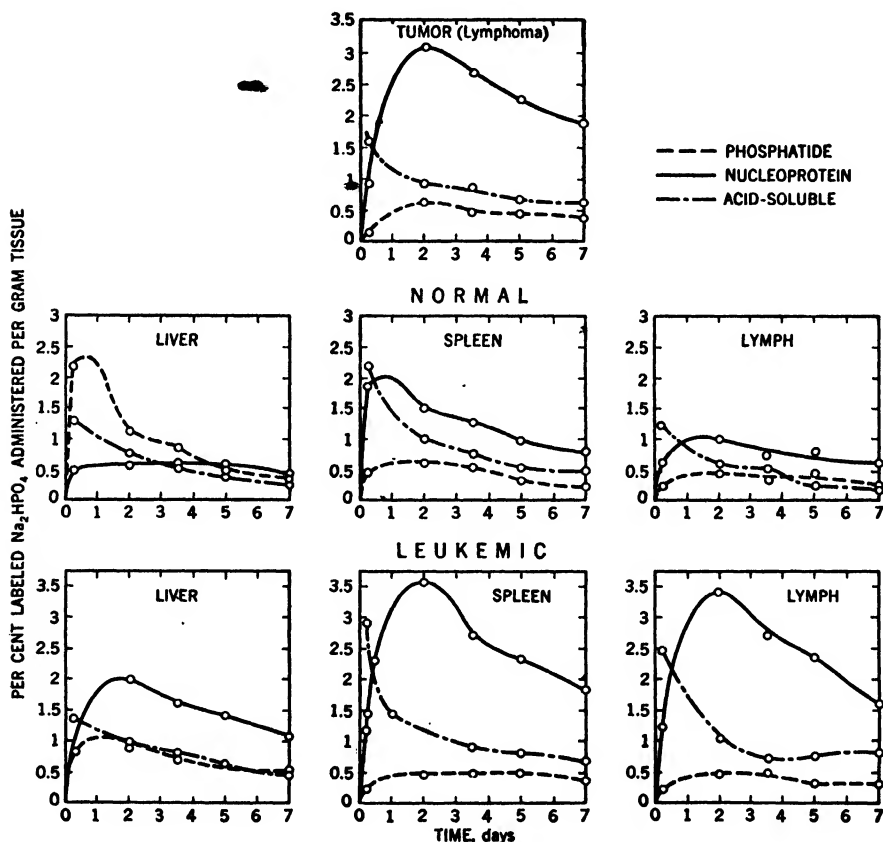


Fig. 18. Comparison of the rates of formation and distribution of the different organic phosphorus fractions in tumor tissue, and in normal and leukemic tissues (178).

acid-soluble, phosphatide, and nucleoprotein fractions of tissues of normal and leukemic mice (178). Some of the results of these studies are shown in

Figure 18. The residual (nucleoprotein) fraction from tumor cells and from leukemic tissues contain considerably more radiophosphorus than do normal structures. These results indicate that nucleoproteins are synthesized at a more rapid rate by leukemic and tumor cells than by normal tissue.

### VII. Intravenous Transfer of $^{32}\text{P}$ from Chromatin to Hepatic Tissue

Marshak and Walker (124) injected labeled chromatin into the blood of rats weighing 50 g. after partial hepatectomy and found the liver nuclei retained much more  $^{32}\text{P}$  than after injection of labeled inorganic phosphate of the same activity (see Table XLVI). The difference between uptake

TABLE XLVI  
 $^{32}\text{P}$  IN REGENERATING LIVERS THREE HOURS AFTER INTRAVENOUS INJECTION OF LABELED SUBSTANCES (124)

Material injected	Number of rats	$^{32}\text{P}$ /g. liver as per cent dose	Standard error of mean
Inorganic phosphate	84	4.52	0.083
Inorganic phosphate	10	4.36	0.084
Rat chromatin	19	26.30	0.670
Lipide (rat chromatin)	15	32.20	0.390
Fat-free chromatin (rat)	5	7.10	0.480
Adenosine triphosphate	7	4.43	0.320
Rabbit chromatin	8	23.10	0.200
Rabbit chromatin soluble in 1 M NaCl	8	16.20	0.470

#### $^{32}\text{P}$ UPTAKE BY NUCLEI

Substance injected	Per cent dose/g. nuclei	Per cent liver $^{32}\text{P}$ in nuclei
Inorganic phosphate	1.54	2.1
Rat chromatin	5.08	1.2
Rabbit chromatin	5.06	1.3
Rabbit chromatin soluble in 1 M NaCl	4.23	1.6
Fat-free chromatin (rat)	5.45	4.6
Lipide (rat chromatin)	5.15	1.0

of  $^{32}\text{P}$  by nuclei after administration of chromatin and inorganic phosphate is much less striking if the  $^{32}\text{P}$  content of the nuclei is compared with the  $^{32}\text{P}$  content of the liver tissue. This comparison seen in Table XLVI shows that the liver tissue takes up a much larger percentage of the labeled chromatin than of the labeled phosphate. This is another example of the enhanced uptake of phosphorus compounds foreign to the plasma by the



liver. If the labeled chromatin splits off inorganic labeled phosphate by enzyme action in the liver, we would expect enhanced formation of labeled compounds in the nuclei corresponding to the high  $^{32}\text{P}$  level maintained in the liver.

That labeled desoxyribonucleic acid intravenously injected into rats weighing 100–200 g. rapidly splits off labeled phosphate in liver was shown by Ahlström and co-workers (7). Some of their results are seen in Table XLVII. After the lapse of two hours an appreciable part of the labeled phosphorus is found in the liver, and more than three-fourths in the acid-soluble fraction.

TABLE XLVII

$^{32}\text{P}$  OF INJECTED LABELED DESOXYRIBONUCLEIC ACID TAKEN UP BY VARIOUS TISSUES AFTER TWO HOURS (7) \*

Rat	Plasma total P	Liver inorganic P	Liver acid- soluble P	Liver total P	Spleen total P	Kidney total P	Plasma inorganic P
I	2.2	11.0	14.5	18.0	0.79		0.81
II	8.8	5.6	7.4	9.4	0.48		0.34
III	1.7	8.2	10.6	14.3	0.92		0.57
IV	1.9	8.6	12.1	16.0	0.65		0.58
V	1.1	6.8	9.2	13.8	0.66	2.80	0.56
VI	1.0	3.7	5.5	8.7	0.38	1.46	0.30
VII	0.32	4.3	6.2	9.0	0.44	1.39	0.25
VIII	0.78	6.1	8.7	10.4	0.77	2.04	0.37
IX blood	3.5	10.2	13.4	20.7	0.85	4.7	
X blood							
XI blood	2.2	9.5	11.9	18.8	0.75	3.7	
XII blood							

\* Values are in per cent of injected  $^{32}\text{P}$ .

In experiments in which liver slices were incubated in bicarbonate-Ringer solution containing labeled desoxyribonucleic acid, the phosphate group of more than two-thirds of the nucleic acid added was found to be split off in the course of four hours, four-fifths of the total acid-soluble  $^{32}\text{P}$  being present in the phosphate fraction. The fact that, in contrast to the phosphate group of desoxyribonucleic acid introduced into the circulation, phosphate of the desoxyribo acid already present in liver cells is not split off at an appreciable rate, may be due to the highly polymerized and shielded state in which nucleic acid exists in nuclei (7).

Not only nucleic acid, but also other organic phosphorus compounds administered give off inorganic phosphate. Choline phosphate appears for

example within a short time as inorganic phosphate in the circulating blood of the rat following intraperitoneal injection of the ester (147). Triphenyl phosphite was found to be decomposed readily after being injected intraperitoneally (8). Phosphatides which are normal constituents of plasma are not decomposed in the circulation at an appreciable rate.

While it is probable that most of the labeled phosphate in nuclei after intravenous injection of labeled chromatin was carried into the nuclei as free phosphate, the possibility of incorporation of some nucleoprotein into nuclei cannot be excluded. Griffith (65) and Avery and co-workers (17) have shown that pneumococci which, in artificial culture, have lost the capsules endowing them with virulence and containing the specific polysaccharides have reverted to avirulent nonspecific types. Such degenerate, nonspecific pneumococci, from whatever specific type derived, could be induced, by cultivation in a medium prepared from a complete virulent type, to reacquire a capsule conferring the corresponding specificity.

Recently Avery and his colleagues were able to isolate and to characterize a chemical principle acting in minute dosage as the specific stimulus to such a transformation. The substance inducing the transformation was found to be a nucleic acid of the desoxyribose type. It is possible that this substance as such enters the nucleus and that we meet here one of the very few cases in which the organism avails itself of phosphorus compounds of high molecular weight of exogenous origin in fundamental synthetic processes.

### VIII. Phosphorus Turnover in Yeast

When bakers' yeast cells (*Saccharomyces cerevisiae*) were suspended for 1.5 hours at 22°C. in (a) glucose-salt, (b) fluoride-glucose-salt, or (c) sustaining salt media, to each of which radiophosphorus in form of phosphate had been added, Lawrence *et al.* (112) found radioactivity only in those cells suspended in the first solution. The uptake of radiophosphorus varied with the concentration of glucose.

By use of ice-cold 5% trichloroacetic acid and hot ether-alcohol solutions, it was determined that approximately 80% of the activity was found in the acid-soluble and 20% in the nucleoprotein fractions. (Approximately 40% of the activity of the nucleoprotein fraction was present in the nucleic acids.) Iodoacetic acid decreased while cyanide increased the rate of uptake of radiophosphorus in the nucleoprotein fraction. Once the yeast cells had incorporated  $^{32}\text{P}$ , it was not lost by resuspending the cells in a radiophosphorus-free medium. Hevesy *et al.*

(90a, see also 84a) found that yeast grown in a labeled nutrient solution, when kept at 20° for one day in a nonlabeled nutrient solution, lost about 3% of its  $^{32}\text{P}$  content; about one-half of the  $^{32}\text{P}$  lost by the yeast cells was found to be present in the nutrient solution as inorganic, and the other half as organic, acid-soluble phosphorus. Irradiation by ultraviolet rays, not however by an x-ray dose of 15,000 r, appreciably increased the loss of  $^{32}\text{P}$  by the yeast cells.

It was furthermore found by Malm (120) that the uptake of  $^{32}\text{P}$  by cells depends on  $p\text{H}$  of the solution in which the cells are suspended. A maximum uptake takes place with  $p\text{H}$  reaches values of 4-5, while at  $p\text{H}$  7 practically no uptake of  $^{32}\text{P}$  is observed. As  $p\text{H}$  of the cells is not influenced by changes in  $p\text{H}$  of the nutritive solution, this observation suggests that processes going on at the cell surfaces or within the cell boundary play an important part in phosphorus metabolism in yeast.

Lindahl and associates (115a) found recently that radioactive phosphate introduced into the cells of bakers' yeast was incorporated into the coenzyme molecule. The rate of this process was under certain conditions dependent on the rate of metabolism; but the phosphate exchange also took place in the absence of exogenous substrate at low temperature (+4°C.), though at a very slow rate.

## IX. Studies of Virus Reproduction

Despite the fact that the introduction of one unit of a virus within a living cell of a susceptible host is followed by the production of millions of virus units, almost nothing is known about the reproductive process. It seemed possible that preparation and isolation of tobacco mosaic virus containing radioactive phosphorus and inoculation of this virus into the diseased plants with the subsequent extensive multiplication of the virus should provide some information concerning the process of virus reproduction. This line of thought induced Stanley (169) to investigate the radioactivity of Turkish tobacco plants inoculated with labeled tobacco virus. Similar investigations were also carried out by Born and associates (25).

When mosaic-diseased Turkish tobacco plants were fed a nutrient solution containing radioactive phosphorus in the form of disodium phosphate over a period of several weeks, about 30% of the phosphorus taken up by the plants was isolated (by Stanley) in the form of purified tobacco mosaic virus. The tobacco mosaic virus growing, in contrast to the plant, at a rapid rate is bound to take up a large percentage of the labeled phosphate which reaches the plant. While an organ or a substance grown in a labeled

medium becomes labeled throughout, parts of a mature organism became labeled only by interchange, the rate of which varies greatly with the organ and the compound in question.

The virus containing radioactive phosphorus, to a large extent present as a constituent of the ribonucleic acid molecule, was rubbed into the lower leaves of Turkish tobacco plants. After twelve days the lower inoculated and the upper uninoculated leaves were investigated, with the result shown in Table XLVIII.

TABLE XLVIII

DISTRIBUTION OF  $^{32}\text{P}$  IN TURKISH TOBACCO PLANTS TWELVE DAYS AFTER INOCULATION OF LOWER LEAVES WITH 58 MG. LABELED TOBACCO MOSAIC VIRUS (169)

Fraction	Relative activity
Virus isolated from inoculated leaves.....	8.3
All material of inoculated leaves except virus.....	33.4
Virus isolated from uninoculated leaves.....	5.8
All material of uninoculated leaves except virus.....	52.5

Since, in Stanley's experiments (as seen in Table XLIX) most of the radioactivity was found associated with nonvirus components in both inoculated and uninoculated portions of the plants, it was impossible to determine whether or not the small amount of radioactive virus found in the uninoculated portions resulted from movement of the inoculated virus. In view of these results it is exceedingly difficult to distinguish between  $^{32}\text{P}$  taken up by the plant in the form of virus and that taken up in the form of virus disintegration products.

## X. Dynamic State of Body Constituents

Perhaps the most remarkable result obtained in the study of the application of labeled phosphorus and of other radioactive and stable (see Schoenheimer, 160) indicators is the discovery of the dynamic state of the body constituents. The molecules constituting the plant or animal organism are incessantly renewed. In the course of this renewal, not only the atoms and molecules taken up with the food participate, but atoms and molecules located in one organ or in one type of molecule will soon be found in another organ or in another type of molecule present in the same or in

another organ. A phosphate radical taken up with the food may first participate in the phosphorylation of glucose in the intestinal mucosa, soon afterwards pass into the circulation as inorganic phosphate, enter a red corpuscle, become incorporated with an adenosinetriphosphoric acid molecule, participate in a glycolytic process going on in the corpuscle, return to the circulation, penetrate the liver cells, participate in the formation of a phosphatide molecule, and after a short interval enter the circulation in this form, penetrate the spleen, and leave this organ after some time as a constituent of a lymphocyte. We may meet the phosphate radical again as a constituent of the plasma, from which it may find its way into the skeleton. Being incorporated in the uppermost molecular layer of the skeleton, it will have a good chance of being replaced by other phosphate radicals of the plasma or the lymph, but it may also have the good fortune to find a more or less lasting abode in the skeleton. This will be the case when phosphate becomes embedded in a newly formed apatite-like bone crystallite.

There are indications that, in the growing organism, the rate of new formation of molecules is appreciably greater than in a fully grown organism. It was found, for example, that in the liver of four-day old rats beside an appreciable formation of additional desoxyribonucleic acid, a very appreciable renewal of "old" molecules takes place as well. The turnover of desoxyribonucleic acid in the liver of four-day old rats is about twenty times more rapid than the corresponding value found for fully grown rats (3).

## XI. Turnover of Sulfur Compounds

The fate of sulfur compounds in the organism has not yet been investigated so extensively as that of phosphorus compounds. This is due to various factors. Radiosulfur is much more difficult to obtain than radiophosphorus; the radiation emitted by radiosulfur is very soft, most of that from radioactive barium sulfate samples weighing more than five milligrams per square centimeter being absorbed in the preparation. Furthermore, while we can study the turnover of phosphorus compounds by administering labeled phosphate, which becomes incorporated in the various phosphorus compounds present in the organism, we have to administer methionine, cystine, or similar compounds for studying the turnover of sulfur (57,172,177). The animal organism cannot avail itself of administered sulfate in building organic compounds containing sulfur (172) (see also Fromageot, pages 369-407, this volume).

## A. FATE OF ELEMENTARY SULFUR FED TO RATS

In the experiments of Tarver and Schmidt (172, see also 173) normal adult rats are fed with 1 mg. of labeled colloidal sulfur by stomach tube. After the lapse of 48 hours less than 0.3% of the radiosulfur can be detected in the proteins of the liver or other internal organs. About 60% is recovered in the urine and 8% in the feces. When, however, 1 mg. labeled methionine is administered to fasting rats, 56% is found in the proteins (14% in the liver) and 36% is oxidized to sulfate.

## B. FATE OF SULFUR FED AS SODIUM SULFIDE TO RATS

When labeled sulfur was administered as sodium sulfide (containing 1.66 mg. sulfur) by stomach tube to rats, a very large part of the sulfur was absorbed and excreted in the urine. The major portion of the radioactive sulfur in the urine was in the form of sulfate sulfur, both inorganic and ethereal. This result indicates that the rat can oxidize sulfide sulfur to sulfate sulfur and thereby change a relatively toxic form of sulfur to an

TABLE XLIX  
CONCENTRATION OF  $^{35}\text{S}$  IN EXCRETA AND TISSUES OF RATS 24 HOURS AFTER ORAL ADMINISTRATION OF SODIUM SULFIDE CONTAINING  $^{35}\text{S}$  (43)

Tissue	Activity of tissue S (counts $\times 10^{-4}$ per mg. S per min.)	
	Rat I	Rat II
Urine		
Inorganic sulfate S	50.94	45.70
Total sulfate S	54.85	53.85
Total S	73.08	52.45
Feces	23.71	7.52
Gastrointestinal tract	5.13	5.34
Bones	1.43	1.33
Heart	0.82	0.58
Kidneys	0.52	0.48
Spleen	0.42	0.34
Gonads	0.26	0.38
Lungs	0.34	.....
Blood	0.21	0.25
Liver	0.17	0.28
Brain	0.02	0.17
Skin	0.53	0.16
Muscle	0.046	0.036
Hair	0.0003	0.0008

innocuous form. The concentration of labeled sulfur in excreta and tissues of rats 24 hours after oral administration is seen in Table XLIX. Dziewiatkowski (44) succeeded also in showing that some of the radioactive sulfur ( $3 \times 10^4$  counts per minute) administered as sulfide is incorporated in the mercapturic acid synthesized after bromobenzene administration and is also present in the cystine isolated from hair, liver, skeletal muscle, and skin. The amount of cystine synthesized by the process employing sulfide sulfur was found to be very small (see Table L), even though there was an active deposition of protein as indicated by an increase in body weight.

TABLE L

ACTIVITY OF CYSTINE SULFUR ISOLATED FROM RAT TISSUE AFTER INTRAPERITONEAL INJECTION OF SODIUM SULFIDE CONTAINING RADIOACTIVE SULFUR ( $3 \times 10^4$  COUNTS PER MINUTE) (44)

Cystine source	Counts per min. per mg. S			
	Rat A	Rat B	Rat C	Rat D
Hair	50	86	3	4
Skin	22	17	5	25
Muscle	61	166	4	13
Liver	16	17	2	8

### C. RATE OF REPLACEMENT OF PROTEIN SULFUR

Labeled methionine was administered in the studies by Tarver and Schmidt (172). One day after administration of methionine the replacement figures shown in Table LI were found in proteins isolated from the

TABLE LI

EXTENT OF REPLACEMENT OF PROTEIN SULFUR ONE DAY AFTER ADMINISTRATION OF LABELED METHIONINE (172)

Organ	Per cent replacement protein S by labeled S	Organ	Per cent replacement protein S by labeled S
Liver	0.41	Pancreas	0.47
Kidney	0.30	Leg muscles	0.01
Spleen	0.18	Red corpuscles	0.01
Lungs	0.17	Stomach mucosa	0.24
Brain	0.08	Intestinal mucosa	0.81
Thyroid gland	0.15		

tissues of fasting dogs. The most rapid replacement was found (172) to take place in intestinal mucosa and pancreas, the slowest in leg muscles and red corpuscles. How far these low replacement figures are due to a low rate of replacement of the proteins or a low rate of penetration of methionine or an intermediary protein sulfur precursor is not known.

In the protein of another dog killed after the lapse of 30 hours, the sulfur replacement percentage in fibrin was found to be 0.15, in pseudoglobulin and albumin 0.14 and 0.16, respectively. As for liver proteins, the replacement percentage was found to be 0.16. The methionine administered has about the same chance to be present, after the lapse of 30 hours, in liver, fibrinogen, pseudoglobulin, and albumin proteins.

The sulfur present in different liver and plasma protein fractions (pseudoglobulin, euglobulin, albumin 1, and albumin 2) has the same specific activity, while the specific activity of cystine is found to be only two-thirds of the specific activity of methionine. From this result it is apparent that the conversion of methionine sulfur to cystine sulfur is not a slow process. Radioactive cystine is found in liver and kidney proteins.

The introduction of radiosulfur into proteins after methionine feeding indicates a synthesis of peptide bonds. That the opening and reclosing of peptide bonds is a rapid process is shown by numerous investigations by Schoenheimer and co-workers (160), and the above results of Tarver and Schmidt (172) can be interpreted as a further proof of the rapid turnover of tissue proteins. Du Vigneaud and associates (179) applied the stable sulfur isotope  $^{34}\text{S}$  as a tracer. When administering labeled methionine to rats kept on a diet free of cystine, up to 80% of the derived cystine produced in the rats was found to contain sulfur from methionine.

When labeled pseudoglobulin or labeled albumin is added to defibrinated dog plasma, no significant transfer of radiosulfur is found to take place from either the pseudoglobulin or the albumin to the other proteins in the plasma, though the data do not exclude the possibility of some exchange between euglobulin and pseudoglobulin.

Experiments undertaken to determine whether or not methionine sulfur can be converted to taurine sulfur by the dog and the rat tended to show positive results. Taurine containing radiosulfur was isolated from the bile of fistula dogs given radioactive methionine. A large part of the labeled sulfur (as methionine and cystine) is contained in the proteins of fasted bile fistula dogs and rats fed methionine.

An enzyme system, present mainly in liver, forms hydrogen sulfide, pyruvic acid, and ammonia from cystine. The same enzyme system was



found to form, to a less extent, active cystine from inactive cystine and hydrogen sulfide containing radiosulfur. When 76 mg. inactive cystine and 1.53 mg. active hydrogen sulfide were added to the enzyme system by Smythe and Halliday (168), 7.5 mg. cystine was recovered and contained 0.7% of the activity.

The rate of appearance of  $^{35}\text{S}$  in the proteins of egg white was determined by feeding methionine containing radioactive sulfur to hens.  $^{35}\text{S}$  was first found in the egg white on the second day and reached its maximal value on the fourth (173).

#### D. FATE OF PLASMA PROTEINS TAGGED WITH RADIOACTIVE SULFUR

Labeled plasma was obtained by Seligman and Fine (161) on feeding cystine, homocystine, or methionine containing radiosulfur, the highest concentration of amino acids in the plasma protein being obtained with cystine. The largest percentage utilization of amino acid in the production of radioactive plasma protein is obtained with cystine as well. In Table LII data are given for the percentage incorporation of radioactive amino acids into plasma protein.

TABLE LII  
INCORPORATION OF RADIOSULFUR IN PLASMA PROTEINS OF THE DOG (161)

Amino acid	Weight fed, mg.	Highest concn. of amino acid in protein fraction of plasma, mg./ml.	Ingested amino acid	
			Incorporated in plasma proteins, %	Excreted in urine as sulfate, %
<i>L</i> -Cystine	100	0.024	5.2	36
<i>L</i> -Cystine	200	0.046	14.8	19
<i>dl</i> -Homocystine	500	0.0032	0.15	66
<i>dl</i> -Homocystine	50*	0.0031	0.9	7
<i>dl</i> -Methionine	50	0.00034	0.06	18
<i>dl</i> -Methionine	150	0.0000	0.00	14

\* Injected intravenously.

The application of labeled plasma proteins to the study of traumatic shock is made possible by the fact that the labeled proteins disappear from plasma at a low rate, as seen in Figure 19. In the case of plasma protein containing radioactive sulfur, 90% of the radioactive protein was circulating 5 hours after injection, 70% 15 hours after injection, and 45% 48 hours after injection.

In Figure 19 the rate of disappearance of radioactive sulfoproteins and radioactive bromoproteins of the circulating plasma of dogs is seen. Radio-

bromoprotein containing less than 0.1% bromine disappears at a somewhat slower rate from the plasma than does radioactive sulfoprotein. The synthetic methods developed by du Vigneaud and co-workers and by Tarver and Schmidt were utilized in preparing from barium sulfate amino acids containing radioactive sulfur. Cystine was obtained in 17% yield and homocystine in 25% yield (102).

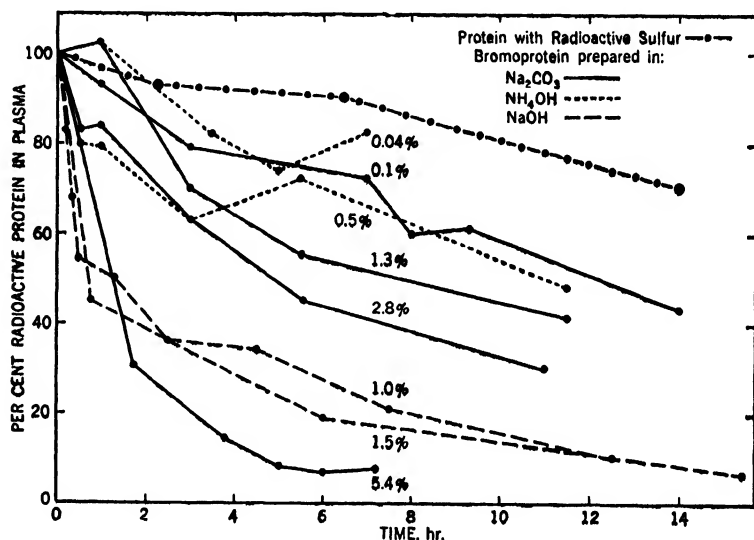


Fig. 19. Rate of disappearance of radioactive sulfoprotein and bromoprotein (containing various percentages of bromine and prepared in several ways) from the circulating plasma of normal anesthetized dogs compared with radioactive plasma protein containing radioactive sulfur (48).

Labeled proteins were also applied in the study of the loss of plasma proteins in hemorrhagic shock (48). A prevailing opinion is that increased permeability of the capillaries exists in shock and that a consequent loss of plasma into tissues ensues such that the effective circulating blood volume falls to a level incompatible with life. This loss of plasma is presumed to occur generally throughout the body as well as in areas of local injury, because replacement of all the plasma or of more than can be accounted for as lost at the site of injury does not sustain the organism. When radioactive proteins were given intravenously to normal dogs and dogs in hemorrhagic shock, both with and without anesthesia, Fine and Seligman (48, see also

162) found no difference in the disappearance curves of the radioactive protein. Tissue analysis of radioactive protein in these dogs gave the same result. However, when the shocked dogs received intravenous infusion in the late or irreversible phase of shock, the radioactivity content of some tissues showed that plasma protein was lost into these tissues in possibly significant quantity.

It is concluded from the experiments described that, while the integrity of capillaries may be impaired in the late shock phase, there is no evidence of significant loss of plasma into tissues in untreated fatal shock following hemorrhage. Hence, an increase in capillary permeability is not a factor in the fatal issue.

Similar experiments were carried out with plasma produced by administering to dogs the amino acid lysine synthesized with heavy nitrogen: 50% of the labeled protein was found to have left the blood stream in about 24 hours; 75% in about six days. Shock due to trauma of intestine or leg was found to show a dilution curve of labeled protein not unlike that of the normal dog (49).

#### E. THIAMIN METABOLISM IN MAN

Thiamin synthesized from radiosulfur was injected intramuscularly in two series of experiments, using a human subject on a normal and on a thiamin-free diet. Determinations of the free (unphosphorylated) thiamin in the urine were made by the thiochrome method, and the radioactive sulfur of the feces and of the inorganic sulfur, ethereal sulfur, and neutral sulfur compounds in the urine were determined.

Rapid destruction of the injected thiamin was indicated in both experiments by the appearance of the radiosulfur in the inorganic fraction of the urine, in amounts increasing to about 15% of that injected daily. No radiosulfur was found in the ethereal fraction. In the neutral sulfur fraction of the urine, the excreted radiosulfur during the period of injections was less than that corresponding to the free thiamin found, indicating rapid interaction of the injected material with that already present in the tissues, and the displacement of pre-existing thiamin. After 36 days on the thiamin-free diet, injection of 8 mg. of radiothiamin over a period of three days resulted in excretion of 0.8 mg. pre-existing thiamin. On discontinuing the injections, destruction of thiamin was again indicated by appearance of quantities of radiosulfur greater than that corresponding to free thiamin.

On the normal diet a total of 61% of injected thiamin was recovered from the urine and 11% from the feces over the period of the experiments.

Of the urinary radiosulfur recovered, 25% represented destroyed thiamin appearing as inorganic sulfate and 18% destroyed thiamin appearing with neutral sulfur compounds (26).

## **XII. Role of Iodine in Thyroid Metabolism. Turnover of Diiodotyrosine and Thyroxine\***

### **A. FORMATION OF LABELED DIIODOTYROSINE AND THYROXINE**

Labeled thyroxine is rapidly formed in the thyroid gland. As early as two hours after administration of a tracer dose of  $^{131}\text{I}$  to rats (140), 1.5 to 3% of it was found by Chaikoff and his associates to be retained as thyroxine in the entire thyroid glands, which weighed 11–21 mg. Increased amounts were found at a later stage. Table LIII records the distribution of the total radioiodine taken up by the thyroid gland of the rat among the thyroxine, diiodotyrosine, and inorganic fractions (see also page 202). Similar results were obtained in experiments with sheep. A larger percentage of the administered  $^{131}\text{I}$  was found as diiodotyrosine than as thyroxine at all stages. Despite a good deal of fluctuation in the actual amounts of labeled iodine deposited as thyroxine and diiodotyrosine in the gland, the proportion of the total labeled iodine represented by each of the fractions remained fairly constant at each time interval. These results suggest that diiodotyrosine is a precursor of thyroxine.

Exposure of rats to cold (0–2°C.) for various periods produces thyroid stimulation, which is definite after 7 days, reaches a maximum after 26 days, but disappears after exposure for 40 days (113). At the time of maximum stimulation by cold, the fixation of  $^{132}\text{I}$  by the gland is 2.7 times that in the controls. Separation of the iodine fractions of thyroid at various times indicates that the turnover of thyroxine and the excretion of iodinated products are increased to about twice the normal rates.

### **B. EFFECT OF THYROTROPIC HORMONE ON TURNOVER RATE**

A thyrotropic preparation was subcutaneously administered to guinea pigs once daily for four days. On the fifth day the hormone-treated as well as the control guinea pigs received intraperitoneally a trace dose of radioiodine. Larger amounts of  $^{131}\text{I}$  appeared in the hyperactive than in the normal animals; in addition, the former contained about twice as

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\* Investigations on the absorption, circulation, distribution, and excretion of radioiodide are discussed in the monographs of Salter (155) and the author (84).

TABLE LIII  
ADMINISTERED RADIOIODINE TAKEN UP BY THE THYROID OF THE RAT (140)

Time after <sup>131</sup> I administration, hr.	Weight of glands, mg.	Per cent of administered <sup>131</sup> I recovered in whole thyroid gland					Per cent of total thyroid <sup>131</sup> I found as		
		Total determined (1)	As thyroxine (2)	As diiodo- tyrosine (3)	As inorganic (4)	Total recovered (2) + (3) + (4)	Thyroxine	Diiodo- tyrosine	Inorganic
2	14.8	10.8	1.56				14.4		
2	17.1	17.3	3.18				18.4		
2	16.1	12.9	1.73				13.4		
2	20.9	15.3	1.94				12.7		
4	19.3	25.7	4.73	17.40	2.08	24.2	18.4	67.7	8.1
4	15.8	16.2	3.52	11.30	1.41	16.2	21.7	69.8	8.7
4	18.5	13.4	2.47	9.80	1.21	13.5	18.4	73.1	9.0
4	16.5	10.5	1.68	7.39	0.90	9.9	16.0	70.4	8.6
48	11.1	32.4	9.82	17.90	3.18	30.9	30.6	55.2	9.8
48	15.2	28.6	9.63	15.60	3.74	29.0	33.7	54.4	13.1
48	18.8	10.7	3.25	6.70	1.02	10.9	30.4	62.7	9.5
48	19.1	52.2	15.80	31.80	4.17	51.8	30.3	60.9	8.0
96	13.2	24.6	5.61				22.8		
96	10.5	37.3	8.85				23.7		
96	13.5	14.0	3.64				26.0		
96	13.6	15.7	4.04				25.7		

much organically bound labeled iodine as did the normal glands, as is seen in Table LIV (see also earlier investigations, 70, 77, 114, 155). Thus, despite the fact that the thyroid gland is known to be depleted of its iodine and thyroxine content under the influence of the hormone, the capacity of the hyperactive gland for removing recently injected iodine and converting it to organic compounds is greatly increased above normal. That thyrotropic hormone produces a prompt and early acceleration in the rate at

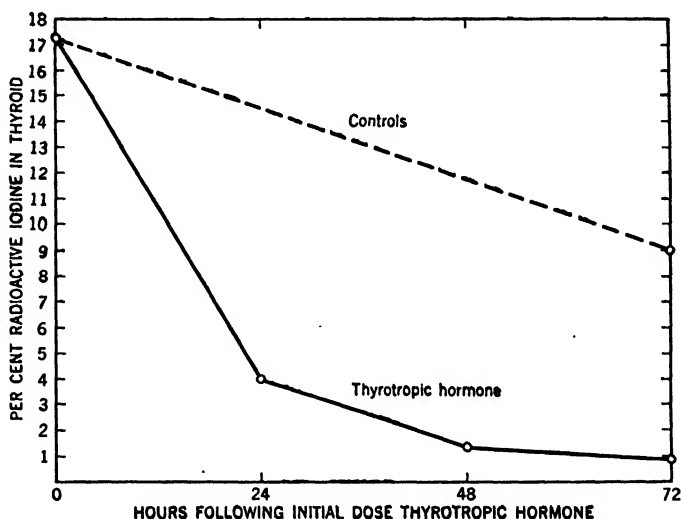


Fig. 20. Effect of thyrotropic hormone on loss of iodine previously stored in thyroid of the chicks (105).

which iodine previously stored in the thyroid of the chick was lost from it is seen in Figure 20.

The plasma of the hormone-treated animals contained larger amounts of organically bound labeled iodine than did the plasma of the control animals, which is just what we should expect (132). After twenty hours about 80% of the plasma iodine in the animals treated with thyrotropic preparation was present as thyroxine. This observation also suggests that the end product of iodine synthesis is thyroxine and beautifully demonstrates the role of thyrotropic hormone in providing thyroxine to the circulation. Hypophysectomy (*cf.* 35) is followed by a reduced uptake of radioiodine by the thyroid gland (115).

TABLE LIV  
RECOVERY OF TRACE DOSE OF LABELED IODINE IN THYROID AND PLASMA  
OF GUINEA PIGS (132)

Experimental condition	Time, hr.	Per cent of radioiodine recovered in thyroid gland as			Per cent of radioiodine recovered in 20 ml. plasma as		
		Thyroxine	Diiodo-tyrosine	Inorganic	Thyroxine	Diiodo-tyrosine	Inorganic
Normal	2	0.44	4.52	0.40	0.025	0.206	2.55
	6	0.58	7.31	0.93	0.012	0.215	1.46
	26	0.97	11.8	0.88	0.17	0.015	0.12
Hyperactive	2	2.38	5.95	3.3	0.15	1.14	1.87
	6	4.23	12.3	4.7	0.19	0.077	0.65
	26	7.17	19.0	3.7	0.38	0.041	0.06

### C. EFFECT OF THIOCYANATE AND THIOURACIL

In thyroids of rats made goitrous by potassium thiocyanate and by thiouracil the uptake of radioactive iodine was found to be reduced.

Two weeks after the animals were placed on a basal diet, a group of rats were given 0.1% thiouracil in the drinking water. Another group was given 0.25% potassium thiocyanate in the drinking water. The remaining 25 animals were maintained on the basal diet alone. On the twenty-eighth day after starting the thiouracil and thiocyanate treatment, all of the animals were injected intraperitoneally with 1 ml. solution containing 2.5  $\mu$ g. labeled iodide. Four hours later all of the animals were killed. The uptake of radioiodine by thyroid found by Rawson *et al.* (145) is seen in Table LV.

TABLE LV  
ADMINISTERED RADIOIODINE IN THYROID GLANDS OF RATS (145)

Group of animals	Mean thyroid weights, mg.	Mean uptake of administered radioactive I, %
Control	15.9	55.7
Thiocyanate treated	29.3	86.5
Thiouracil treated	49.5	10.1

It is evident from the above figures that potassium thiocyanate and thiouracil have a goitrogenic effect. In the hyperplastic thyroids produced by thiouracil the uptake of radioiodine is appreciably lower than in the controls, while the opposite is the case for the thiocyanate-treated rats. While the uptake of  $^{131}\text{I}$ , by the control thyroids, was 56% of the administered tracer dose, that by thyroids of thiocyanate-treated rats, 87%, and that by thiouracil-treated, 10% (145). In other experiments the daily feeding of 3.4 to 4.2 mg. of thiouracil for seven days was found to depress the up-

take of injected  $^{131}\text{I}$  by the thyroid gland of the rat. The recovery of the gland as judged by its capacity to remove injected radioiodines, was complete in two weeks (56).

Thiouracil was also found to inhibit uptake of radioactive iodine by thyroid of normal chick and by hyperplastic chicks (111). Administration of thiourea, adding to the diet 1% of thiourea for one month, was found to obstruct the accumulation of radioiodine in any form in the thyroid of the rat (107).

#### D. FORMATION OF DIIODOTYROSINE AND THYROXINE IN THYROIDECTOMIZED ANIMALS

Does formation of diiodotyrosine and thyroxine take place in thyroidectomized animals? This point has been investigated by Morton *et al.* (130) making use of rats thyroidectomized at the age of four to six months and maintained on a stock diet for two to eight months. Their basal metabolic rate was 40–50% below normal. Tracer doses of  $^{131}\text{I}$  were administered and the various organs analyzed for their thyroxine, diiodotyrosine, and inorganic iodine content. All organs investigated were found to contain these compounds, as is seen in Table LVI, the small intestine containing a much larger amount of radiothyroxine after 24 than after 2 hours. As early as 96 hours after its injection, 30% of the radioiodine contained in the liver and in the small intestine were organically bound, 20% as diiodotyrosine and 8% as thyroxine. The ratio between inorganic and organic iodine was, however, much larger in the different organs than in the case of nonthyroidectomized rats.

Thyroxine and diiodotyrosine extracted from the organs were recrystallized five times. In each recrystallization of thyroxine, 10 mg. of nonradioactive diiodotyrosine were added in order to wash out any contaminating radiodiiodotyrosine by dilution. Such a washing-out process is much used in the purification of labeled compounds. In the case of diiodotyrosine recrystallization, 10 mg. of nonradioactive thyroxine were added each time for the same purpose.

The specific activities of both thyroxine and diiodotyrosine did not change significantly after the second recrystallization. This would be expected to occur only in a case in which the substance giving the radioactivity was identical with the material that underwent repeated recrystallization.

The completeness of thyroidectomy was checked with great care by histological examination of serial sections of the whole neck and chest regions of the animals, and in some cases by a radioautographic method in which the tissues are rolled out and exposed to an x-ray film. Under such



TABLE LVI  
FORMATION OF THYROXINE AND DIODOTYROSINE BY THYROIDECTOMIZED RATS (130)

Rat No.	Body weight, g.	Interval after thyroidectomy, month	Oxygen consumption		Tissue	Time after injection, hr.	Administered <sup>131</sup> I recovered per g., %	<sup>131</sup> I of tissues found as		
			Per sq. meter per 24 hours	Decrease below normal, %				Thyroxine, %	Diodotyrosine, %	Inorganic, %
131	395	8	97	47	Small intestine	2	0.132	1.5	13.6	87.4
132	385	2	96	48	Small intestine	2	0.143	1.5	9.4	90.0
131					Liver	2	0.0733	1.9	12.6	88.4
132					Liver	2	0.0648	1.7	11.4	85.1
134	290	4	92	50	Small intestine	24	0.110	1.9	20.1	78.4
137	310	8	108	41	Small intestine	24	0.0494	3.9	19.9	69.6
134					Liver	24	0.0692	1.8	20.5	77.5
137					Liver	24	0.0444	4.5	15.2	80.4
134					Muscle	24	0.0408	1.3	8.1	88.0
137					Muscle	24	0.0190	3.3	12.0	83.1
134	285	2	109	41	Plasma	24	0.212	1.0	7.01	91.8
135					Plasma	24	0.126	0.8	12.2	86.9
136	415	8	101	45	Small intestine	96	0.00583	8.0	13.0	74.7
137	375	2	95	48	Small intestine	96	0.00861	7.2	15.0	77.4
140	415	2	105	43	Liver	96	0.00563	5.8	21.2	73.7
141					Liver	96	0.00957	8.6	21.3	69.9

circumstances a high concentration of radioactive iodine such as occurs in residual fragments of thyroid tissue is automatically revealed. Finally, the results of the experiments were not in any way influenced by previous hypophysectomy as would have been expected if they had been due to undetected thyroid tissue. The thyroidectomy was carried out very carefully and controlled by radioautographs. Chaikoff and co-workers also found that the administered  $^{131}\text{I}$  can be recovered almost quantitatively from the tissue.

At first sight it may be astonishing to find the formation of thyroxine in a thyroidectomized organism, but this is in no way surprising in view of the discoveries of Mutzenbecher (133) and others. By iodination of casein, they obtained preparations that were physiologically active and from which thyroxine could be isolated. Harington and others obtained similar results (72).

#### E. FORMATION OF DIODOTYROSINE AND THYROXINE IN TISSUE SLICES

That the thyroid gland has an extraordinary capacity for accumulating iodine is also demonstrated by experiments with tissue slices. At the end of two hours incubation in bicarbonate-Ringer solution containing tracer doses of radioiodide 81% of the  $^{131}\text{I}$  was recovered in 300 mg. surviving slices of thyroid gland of sheep, but only 2% in slices of liver, as is seen in Table LVII (164). Homogenization or desiccation of the tissue was

TABLE LVII  
ACCUMULATION OF  $^{131}\text{I}$  IN ONE HOUR IN SURVIVING LIVER AND THYROID SLICES  
OF SHEEP (164)

Tissue	Per cent of Ringer $^{131}\text{I}$ recovered in slices as		
	Organic	Inorganic	Total
Liver	0.1	2.6	2.7
Liver	0.0	2.1	2.1
Liver	0.1	2.2	2.3
Thyroid	57.5	21.5	79.0
Thyroid	56.9	23.1	80.0
Thyroid	63.4	21.9	85.3

found by Chaikoff and his associates to prevent the conversion of radioiodine to diiodotyrosine and thyroxine (129). The process of conversion is retarded by poisons known to inhibit aerobic oxidation involving the cyto-

chrome oxidase system (potassium cyanide, hydrogen sulfide, carbon monoxide, and sodium azide) (see Table LVIII). Anaerobiosis causes a strong inhibition when the amount of oxygen is reduced below 0.6% (106). The

TABLE LVIII

EFFECT OF THIOURACIL ON ACCUMULATION OF  $^{127}\text{I}$  OF RINGER SOLUTION BY SURVIVING THYROID SLICES (55) \*

Compound added	$^{127}\text{I}$ of Ringer solution recovered in slices as			$^{127}\text{I}$ of Ringer solution incorporated into slices as		
	Organic, %	Inorganic, %	Total, %	Organic, %	Inorganic, %	Total, %
None	28.4	56.2	82.6	2.64	5.62	8.26
None	22.9	50.0	72.9	2.29	5.00	7.29
None	13.6	54.0	67.6	1.36	5.40	6.76
$10^{-3}$ M thiouracil	2.4	75.5	77.9	0.24	7.55	7.79
$10^{-2}$ M thiouracil	2.3	65.2	67.5	0.23	6.52	6.75
$10^{-3}$ M thiouracil	2.0	59.8	61.8	0.20	5.98	6.18

\* 300 mg. of slices were incubated for one hour at  $38^\circ$  in a bicarbonate-Ringer medium containing inorganic iodide,  $^{127}\text{I}$ , and 10% of inorganic iodide,  $^{127}\text{I}$ . The slices were then separated from their medium and analyzed.

TABLE LIX

EFFECT OF CARBON MONOXIDE ON THYROXINE AND DIIODOTYROSINE FORMATION (156) \*

Expt. No.		Gas mixture above Ringer solution				Per cent of Ringer $^{127}\text{I}$ recovered as		
		$\text{O}_2$ , %	$\text{CO}_2$ , %	$\text{CO}$ , %	Remarks	Thyroxine	Diiodotyrosine	Inorganic iodine
1	Control	95	5	0		12.1	51.9	36.0
						12.4	56.9	30.7
						11.5	47.0	41.5
	CO	Trace	5	95	In dark	1.4	6.0	92.6
						2.0	6.0	92.0
						3.9	11.5	84.6
	CO	Trace	5	95	In light	3.4	6.8	89.8
						3.9	19.3	76.8
						2.6	7.8	89.6
2	Control	95	5	0		7.7	80.5	11.8
						6.0	83.4	10.6
						7.9	83.2	8.9
	CO	5	5	90	In dark	1.8	15.0	83.2
						2.6	21.0	76.4
						1.8	10.4	87.8
	CO	5	5	90	In light	4.5	57.0	38.5
						4.8	54.5	40.7
						2.3	40.3	57.4

\* Each flask contained 300 mg. of slices of sheep thyroid in 3 ml. of bicarbonate-Ringer solution; period of incubation two hours.

inhibition by carbon monoxide is more pronounced in the dark than in the light (see Table LIX).

In order to study the mechanism of action of various known goitrogenic compounds, Chaikoff and colleagues investigated effects of the compounds on *in vitro* conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine by thyroid slices as well. Thiouracil (see Table LVIII) and allylthiourea were found to be effective inhibitors even at concentrations as low as  $10^{-4}$  M (55). Similar effects are produced by sulfanilamide, sulfapyridine, sulfaguanidine, and sulfathiazole. These substances, at a concentration of  $10^{-3}$  M, inhibit the formation of radioiodotyrosine and radiothyroxine (54). Numerous compounds structurally related to the sulfonamides and aminobenzoic acid were tested for their effects on the *in vitro* conversion of radioactive iodide, to thyroxine and diiodotyrosine by surviving tissue slices. A free aromatic amino or hydroxyl group was found to favor inhibitory activity, while the presence of such groups as the sulfonamide, the sulfonic acid, or the carboxyl was found to be unrelated to activity. The results obtained indicate a correlation between ease of oxidizability and inhibitory activity among the compounds tested (175).

When, instead of adding tracer doses of radioiodine to the Ringer solution, 10  $\mu$ g. or more was added, an inhibition of the formation of labeled thyroxine and diiodotyrosine in slices of sheep thyroid at the expense of inorganic iodide of the medium was observed, as seen in Table LX.

TABLE LX  
INHIBITORY EFFECT OF ADDED INORGANIC IODINE ON FORMATION OF LABELED  
THYROXINE AND DIIODOTYROSINE (131)

Thyroid tissue in flask, mg.	Inorganic iodide $^{127}\text{I}$ added to flask, $\gamma$	Per cent of Ringer $^{131}\text{I}$ recovered as			Ringer $^{131}\text{I}$ converted to	
		Thyroxine	Diiodotyrosine	Inorganic	Thyroxine, $\gamma$	Diiodotyrosine, $\gamma$
300	0.3	10.6	45.6	43.8	0.03	0.14
300	0.3	8.7	42.9	48.4	0.03	0.13
305	20.3	2.2	6.1	91.7	0.45	1.2
305	20.3	2.1	6.9	91.0	0.43	1.4
305	50.3	0.50	2.4	97.1	0.25	1.2
300	50.3	0.40	2.4	97.2	0.20	1.2

The inhibiting effect of iodine on conversion of inorganic iodide to thyroxine and diiodotyrosine is possibly due to iodination of the enzymes concerned.

Cyanide and sulfide, in addition to completely inhibiting formation of thyroxine and diiodotyrosine, markedly depress accumulation of  $^{131}\text{I}$  by thyroid slices. Despite the almost complete inhibition of thyroxine and

diiodotyrosine formation by  $10^{-3}$  M azide, as much as 60% of the Ringer solution iodide entered the tissue slices. Sulfanilamides, which strongly inhibit the *in vitro* conversion of Ringer iodide to thyroxine and diiodotyrosine, have at a concentration of  $10^{-3}$  M little effect on the iodine-concentrating capacity of thyroid tissue. From these and similar facts Chaikoff and co-workers (157) conclude that in the thyroid tissue there exists a mechanism for concentrating iodine that does not depend upon the conversion of inorganic iodide to thyroxine and diiodotyrosine.

Biological iodination was found to take place in 5 ml. unpasteurized milk to which, beside 0.5  $\mu$ g. radioiodide, a phosphate buffer and 0.45 mg. xanthine were added. In the course of 45 minutes at 38°C. about half the iodide added was no longer present in the inorganic iodine fraction. The presence of thiourea inhibits the effect of xanthine (106).

After it had been found, by making use of radioiodine as a tracer, that diiodotyrosine and thyroxine were formed in tissue slices placed in bicarbonate-Ringer, the formation of these compounds was also demonstrated by Harington (72) with the usual analytical methods. He found that both diiodotyrosine and thyroxine were formed in the presence of iodine; but, in the absence of iodine in a medium containing diiodotyrosine and thyroid slices, no increase of thyroxine could be observed. This suggests that the essential biochemical reaction may be the liberation of iodine from iodide by an oxidizing enzyme system. The enzymic reaction is concerned with the formation of diiodotyrosine.

#### F. FATE OF INJECTED LABELED THYROXINE

Joliot and associates (97,98,115,171) applied the methods of Harington and Barger in the preparation of labeled thyroxine and compared uptake

TABLE LXI

ACTIVITY FIGURES FOR THE UPTAKE AND EXCRETION OF RADIOIODINE BY RABBITS AFTER ADMINISTRATION OF LABELED SODIUM IODIDE AND LABELED THYROXINE (98)

Organ	11.7 mg. labeled I injected as NaI			7.8 mg. labeled I injected as thyroxine		
	Iodide	Diiodo-tyrosine	Thyroxine	Iodide	Diiodo-tyrosine	Thyroxine
Urine	13.4	10	0.1	5.6	8.3	8.25
Thyroid	312.5	59	2.9	20.9	8.5	1.7
Fetus	7.2	7	1.1	0.23	0.35	0.45
Blood	2.3	2.5	0.2	0.42	0.5	1.5

and excretion of labeled thyroxine injected intravenously to rabbits with behavior of injected labeled iodide. Through the bile up to fifty times as much labeled thyroxine iodine as labeled iodide is found to be excreted. The thyroid gland takes up appreciably more radiolodine after administration of radioiodide than after administration of radiothyroxine, as may be seen in Table LXI. The uptake of labeled thyroxine per gram of hypophysis tissue was found to be larger than the uptake of labeled iodide. Also a greater percentage per gram hypophysis tissue of the radiolodine administered as thyroxine is taken up than of administered inorganic radiolodide.

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# BACTERIAL LUMINESCENCE

By FRANK H. JOHNSON,\* *Princeton, N. J.*

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## I. Introduction

Bioluminescence, in its manifold aspects, has been treated comprehensively and in detail in the recent monograph (99) and review (100) by

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\* Fellow of the John Simon Guggenheim Memorial Foundation, 1945-1946.

Harvey, published in 1940 and 1941. The present report deals largely with the subsequent advances, with particular reference to the luminescence of bacteria.

As with chemiluminescence in general, light emission by living organisms and their products depends on an oxidative reaction which furnishes the energy for excitation of the molecule that emits. Dubois (58) first showed that it is possible to extract from the photogenic material of certain organisms a relatively heat-stable substance, *luciferin*, and a heat-labile substance, *luciferase*, which give rise to luminescence when mixed in aqueous solution. The luciferase was interpreted as a specific oxidative enzyme, acting upon luciferin as its substrate (59). Through the studies of Harvey and his associates much progress has been made in the purification of luciferin from the ostracod, *Cypridina*, and, although a final identification has not yet been achieved, significant properties of the molecule, as well as of the light-emitting system, have been established.

Apart from a few experiments of uncertain interpretation (77) luminous extracts thus far have not been obtained from bacteria. The luminescent system in these organisms, like that of many other oxidative systems in bacteria, appears to be intimately associated with intact cells (138). By analogy, and from similarities in properties of the bacterial to other bioluminescences from which extracts have been prepared, however, it is generally assumed that a fundamentally similar mechanism is concerned.

An important aspect of the problem of bacterial luminescence is the separation and purification of the reactants in the light-emitting system. The other most important aspects consist in, first, the elucidation of the relation of the luminescent oxidation to other metabolic processes involved in the total respiration, and second, the physical chemistry of the luminescent reaction, especially with reference to the mechanism of rate control, and the information thereby provided with respect to the control of biological reactions in general. The most extensive work of the past five years has been carried out in regard to this latter aspect. As a result, a rational interpretation has been made possible concerning the quantitative effects of temperature, hydrostatic pressure, hydrogen-ion concentration, and certain inhibitors of this system, including sulfanilamide and narcotics of the lipide-soluble series, both separately and in relation to each other. In studies of this kind luminescence possesses unique advantages, for no other enzyme system has such an instantaneous, readily and accurately measurable index to its own reaction velocity. Cogent evidence indicates that in

luminescence the brightness of the light, under given conditions, is proportional to the velocity of the oxidative reaction.

## II. Nature of the Reactants

### A. PROPERTIES OF *CYPRIDINA* LUCIFERIN

Crude luciferin is readily obtained by extracting the dried and ground whole organisms of *Cypridina* with water at about 100°C., and immediately cooling. Crude luciferase is obtained by extracting similar material with water at room temperature. Luminescence appears when the cool water is added, but soon goes out as the substrate is oxidized. Methods of partial purification of luciferin have been worked out by Kanda (136) and Anderson (4). The latter has obtained highly purified luciferin in a concentration about 2000 times that of the original dried material by extracting first with methyl alcohol, followed by butyl alcohol, and benzoylation. The benzoylated extractives are removed from butyl alcohol in water, which is then extracted with ether. Hydrolysis of the inactive luciferin in the aqueous solution is effected with hydrochloric acid, and the benzoylation-hydrolysis procedure is repeated. The process of hydrolysis is carried out in the absence of oxygen. The product is light yellow in color, and under rigidly anaerobic conditions remains stable for years at room temperature. It immediately gives luminescence when added to the enzyme plus oxygen.

The collected results of experiments with crude and purified luciferin (99,100) indicate that it is a relatively small molecule, soluble in water, dilute acid, alkali and salt solutions, diffusible through cellophane, nonantigenic, and not destroyed by trypsin. It has been estimated that luminescence visible to the dark-adapted eye may be obtained from luciferin in a dilution of 1:40,000,000,000 (94). With regard to some of the properties of luciferin, based on results obtained with crude extracts, it would be desirable to repeat the experiments with the purified material, inasmuch as different results are sometimes observed, *e.g.*, cyanide apparently has no effect on the luminescent oxidation in crude extracts (91,185) but combines irreversibly with the luciferin in purified solutions (82).

Quantitative estimates of luciferin are based on the total amount of light emitted in the presence of the enzyme under standard conditions (3). The light intensity in relation to time, as well as the integrated total luminescence, is readily measured with a photocell whose current is fed into a condenser connected with a Lindeman electrometer. The charge on the condenser is balanced by a potentiometer at frequent intervals during the course of the reaction. Both the kinetics and total luminescence may be greatly influenced by a two-way oxidation of luciferin described below.

In slightly alkaline or slightly acid solutions luciferin undergoes a dark oxidation, spontaneously in the presence of molecular oxygen, or in the presence of certain agents such as ferricyanide or cerium sulfate (5). This reaction is slower in dilute hydrochloric acid or dilute sodium hydroxide.

The product, oxidized luciferin, which does not react with luciferase to give luminescence, decomposes on standing at room temperature. If sodium hydrosulfite is added within a few minutes, however, reduced luciferin is again obtained with little loss, and will give rise to light emission in the presence of the enzyme plus oxygen. Following this luminescent oxidation only a small fraction of the light emitted may be obtained a second time by treating the solution with sodium hydrosulfite and then oxygen. It would thus appear that most of the luciferin is destroyed during the luminescent oxidation, whose product, oxyluciferin, is not identical with the oxidized luciferin of the ferricyanide reaction (5). It has been suggested that two reactions, involving different groups on the luciferin molecule, take place in the dark and luminescent oxidation, respectively (39,133). The former appears to be easily reversible, and data concerning the effects of quinhedrone and other agents indicate that, as a preliminary value, the luciferin system has an oxidation-reduction potential about 0.01 v. negative to quinhedrone at 23°C. and pH 6.8, suggesting a relation of this system to certain naturally occurring sulfhydryl and hydroxybenzene derivatives (5,12,139). The amount of energy available in this oxidation, however, is not sufficient to give rise to luminescence of the wave lengths observed (124,126). The products of the luminescent oxidation may be partially "resynthesized" by treatment with sulfur oxychloride and then with diazomethane and dilute sulfuric acid, which is consistent with the view that the reaction difficult to reverse consists in the destruction of a primary alcohol group. The irreversible combination of purified luciferin with cyanide suggests the presence of a keto group, and on the basis of these considerations a partial structure of the luciferin molecule has been proposed by Chakravorty and Ballentine (39). The same authors failed to detect any nitrogen, sulfur, halogen, or ash in microanalyses of purified luciferin solutions. Such solutions contain acid-labile phosphate that is released during the luminescent reaction, according to McElroy and Ballentine (145), who believe that luciferin contains "energy-rich" phosphorylated molecules, and that the light-emitting reaction depends on phosphorolysis together with oxidation of a keto alcohol side chain, the latter constituting the "irreversible" reaction. Although these authors apparently assume that the molecules which emit are therewith destroyed, the observed phenomena may be accounted for equally well on the assumption that the destruction involves only those molecules which do not emit, whereas those which become excited and do emit are stabilized, with the result that they can be reduced and subsequently participate again in the

luminescent reaction. There is no evidence against the latter view, which is supported by a general principle of theoretical chemistry. The pathways of the reversible and irreversible reactions accompanying luminescence can be most readily understood with the aid of a potential energy diagram (Fig. 3, page 222). Thus, there are three possible fates of the luciferin molecules participating in the reaction: (1) they may undergo excitation, emit, and thereby be reversibly oxidized, (2) they may fail to become excited in this reversible oxidation, or (3) they may be destroyed. The second of these possibilities should not be confused with the reversible, dark oxidation under the influence of ferricyanide, etc.

Ultraviolet light in the region between wave lengths of 2800 to 2300 Å destroys purified luciferin, apparently independently of oxygen, and the same wave lengths inactivate the enzyme (45). The former substance was found to absorb completely below 2500 Å, with maxima at about  $\lambda$  2700, 2900, and 3100 to 3200 Å. The latter absorbs completely at  $\lambda$  2400 Å, and has a maximum at 2800 Å.

Luciferin may be sensitized to visible radiation by the addition of crude, boiled, aqueous extracts of *Cypridina*, by eosin, fluorescein, or riboflavin. Kinetic data with solutions so treated suggest that it is primarily the reversibly autoxidized form of luciferin which is affected.

Spectroscopic studies have furnished significant data concerning the luminescent oxidation. Chase (41,44) has shown that reduced luciferin has an absorption peak at 435  $m\mu$ . When oxygen is added this peak is replaced by one at 465  $m\mu$ , which then disappears leaving an almost colorless solution. In the ultraviolet, bands are evident at about 310 to 320  $m\mu$  and 280 to 260  $m\mu$ , with complete absorption below 240  $m\mu$ , in agreement with earlier observations (45). During nonluminescent autoxidation the absorption decreases in the region of 250 to 320  $m\mu$ , and subsequently increases in the region between 320 and 400  $m\mu$ .

When luciferase together with oxygen is added to reduced luciferin, and the oxidation proceeds with light emission, the same changes in the absorption spectrum of visible light take place, but at a rate approximately one hundred times faster than in the dark autoxidation. It is evident, therefore, that the luciferin undergoes the same changes in both cases. The presence of the enzyme makes light emission possible to a visible extent. No luminescence of luciferin in water plus oxygen, but without luciferase, has been observed except under certain special conditions. Luminescence, presumably of the luciferin, takes place in crude extracts of *Cypridina* in 95% alcohol at 70°C., a condition under which the enzyme is de-



natured (98). This fact, together with the fairly close correspondence between the emission (65) and absorption spectra (Figs. 1 and 2), indicates that the light-emitting molecule is luciferin. The peak at  $4750 \text{ \AA}$  in the former spectrum evidently corresponds to that at  $4650 \text{ \AA}$  in the latter. The

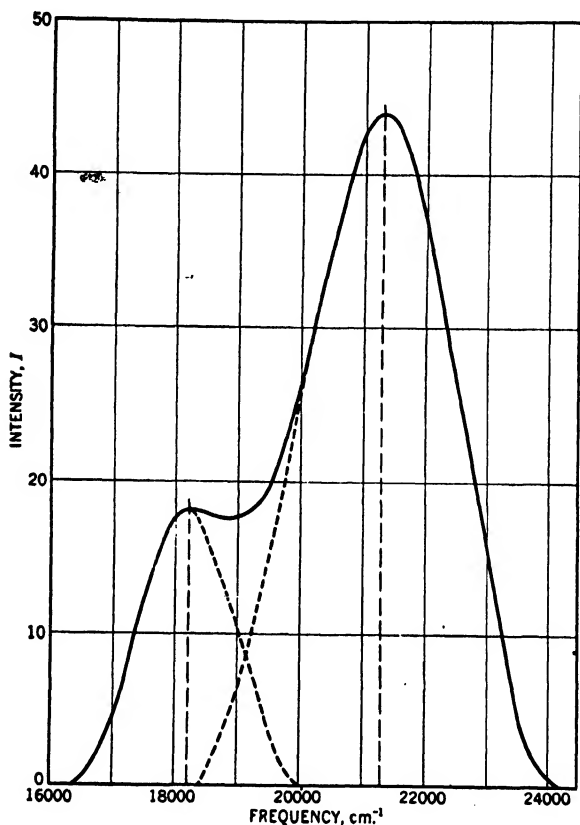


Fig. 1. Intensity-frequency curve of luminescence emitted by crude extracts of *Cypridina* (65).

more rapid destruction of the luciferin in the luminescent oxidation occurs, not because different hydrogen atoms are removed, but because the mechanism is different in the presence of the enzyme.

With the enzyme, the reaction proceeds about one hundred times faster than the autoxidation. In Figure 2, each spectrogram was obtained in from two to four minutes

by means of the Hardy recording spectrophotometer (87). Curves *A* and *G*, immediately after exposing the luciferin solution to oxidation; curves *H*, *I*, *J*, *K*, *L*, *M*, and *N* after 9, 20, 30, 39, 49, 455, and 1140 minutes, respectively, without luciferase; curves *B*, *C*, *D*, *E*, and *F*, at 1, 12, 22, 40, and 457 minutes, respectively, following the addition of luciferase (43).

Based on the foregoing observations, and in part on the mechanism of reactions proposed by Weiss (190), as well as in part upon facts concern-

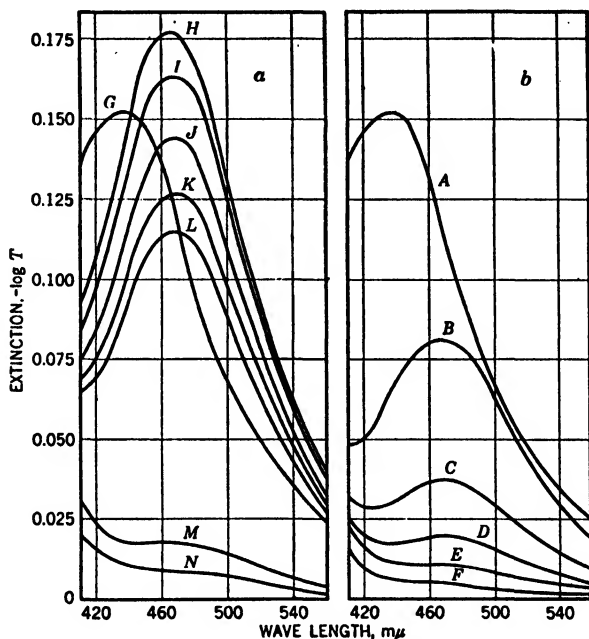
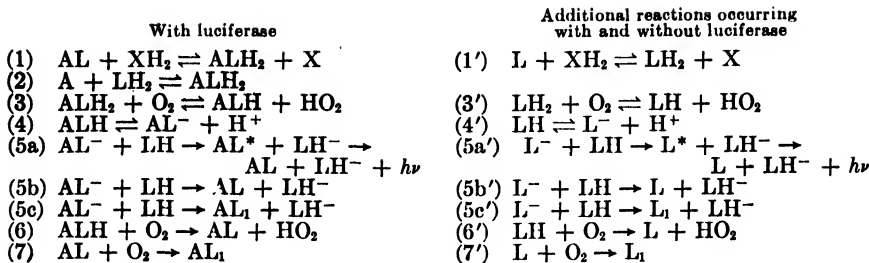


Fig. 2. Changes in absorption spectrum of purified luciferin of *Cypridina* during autoxidation (*a*) without visible luminescence, and during the light-emitting oxidation (*b*) in the presence of luciferase.

ing the luminescence of bacteria and of aminophthalic hydrazide (57), the following scheme has been suggested for the luciferin-luciferase reaction (126). Reduced luciferin is represented by  $\text{LH}_2$  (absorbing at  $435\text{ m}\mu$ ), the oxidation product as  $\text{L}$  (absorbing at  $465\text{ m}\mu$ ), excited luciferin as  $\text{L}^*$ , destroyed luciferin as  $\text{L}_1$ , and luciferase as  $\text{A}$ :



To the left of the activated state the distance between  $AL^*$  and  $LH^-$ , or  $AL^-$  and  $LH$ , or  $AL$  and  $LH^-$ , respectively, is the abscissa. The amount of the attractions between the respective pairs indicated by the minima on the left is not known experimentally. A reaction with oxygen may be involved in passing over the activated state. The middle curve corresponds to the distance between  $AL^- + LH$ , decreasing until, in the dotted region, there is an electron transfer when the system shifts to the upper level, returning to the left as  $AL^*$  and  $LH^-$ .  $AL^*$  may then radiate, becoming nonexcited  $AL$ . On the other hand, instead of forming excited  $AL^*$ , the system may pass irreversibly through the activated state, or it may return unchanged as  $AL^-$  and  $LH$ .

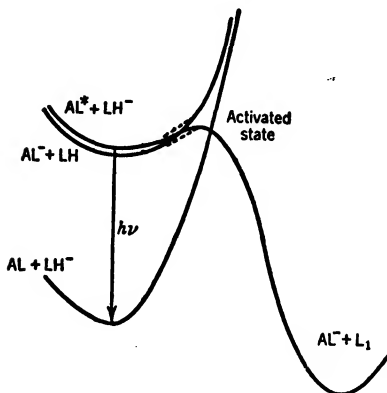


Fig. 3. Diagram illustrating three possible energy states during the oxidation of luciferin (126).

The different states of luciferin in reaction (5) may be represented on a potential energy diagram, as shown in Figure 3.

## B. LUCIFERASE

The luciferase of *Cypridina* has been partially purified by dialysis and elimination of substances precipitated during the process. The product, about fifty times more potent than the original material on a dry weight basis, is in a practically water-clear solution at moderate dilutions (82).

Concentrated solutions, after prolonged dialysis, remain active for at least two years in the refrigerator, and emit light without the addition of luciferin, if treated with sodium hydrosulfite, or certain other reducing agents, then oxygen (124). On the basis of these observations, as well as the available spectroscopic data and the energy changes involved, it has been suggested that the luciferin-luciferase system is possibly a flavopro-

tein, with luciferin as the prosthetic group. In this connection it is perhaps significant that the light-emitting organ of lampyrids contains excessively large concentrations of flavin (11,27). Objections have been raised to these interpretations (7,145). Flavin has recently been isolated from luminous bacteria and appears to be no different from the flavin of other sources (184). This does not exclude the possibility that the luminescent system is a flavoprotein. A possible role of flavin, directly or indirectly, in luminescence is hypothetical and awaits further clarification.

Luciferase processes a high degree of genetic specificity. Although it was once thought that the luciferin and luciferase of closely related species would react to give a luminescence qualitatively similar to that associated with the particular enzyme (92-95), more recent studies (101,126) have suggested other interpretations of the earlier observations, and the spectroscopic evidence makes it difficult to interpret the source of light as other than the luciferin.

### C. KINETICS OF THE REACTION

The reaction is first order with respect to either luciferin or luciferase (2), but complications in the kinetics may arise through the simultaneous light-emitting oxidation and the dark, reversible autoxidation of the luciferin, and possibly other factors. With luciferase in a sufficient concentration that the emission of visible light at room temperature ceases within around five minutes or less, and with luciferin essentially all in the reduced state at the start, the intensity of luminescence is highest almost immediately after mixing the two, then decays logarithmically with time. Toward the end of the reaction, however, there may be a perceptible decrease in the velocity constant, and, if considerable quantities of reversibly oxidized luciferin are initially present with the reduced luciferin, luminescence may decrease rapidly at first, followed by a long period of dim luminescence (5). The latter probably results from a slow reduction, with subsequent luminescent oxidation, of the reversibly oxidized molecules, and indicates a change in the pace-setting reactions that is understandable in terms of the reaction scheme set forth above. Discrepancies in the logarithmic order of decay that have been noted with crude extracts (2,104,177) are absent under carefully controlled experiments with purified materials (46) and apparently result from the presence of impurities which possibly influence the relative rates of oxidation and reduction of the luciferin during the course of the light-emitting reaction.

The logarithmic decay of luminescence conforms to the order of de-

creasing concentration of substrate in an enzyme reaction. This relation, together with the facts that the total luminescence, under favorable conditions, is proportional to the amount of luciferin added, while the velocity constant depends on the amount of luciferase, indicates that the brightness of the light at any moment is proportional to the reaction velocity. This is a very important aspect of the luminescent reaction in kinetic studies, for it means that the reaction has a natural, practically instantaneous index to its own reaction rate. This remains true, of course, even though the rate-determining step may be altered by varying the conditions. For example, at very low concentrations of molecular oxygen, reaction (3) would become limiting, of in mixtures of reversibly oxidized and reduced luciferin, reactions (1) and (2), respectively, may be chiefly the pace setters at different times. According to data obtained with the flow method (40), reaction (3) is considerably faster than reaction (2). When oxygen is added to luciferin and luciferase that have been mixed under essentially anaerobic conditions, it requires 0.002 second for development of half-maximum luminescence intensity, whereas, if the luciferin and luciferase are mixed in solutions each containing dissolved oxygen, it requires 0.006 second. In the former case, the time required is independent of concentration of oxygen added, above about ten millimeters of mercury. In the latter, the time is independent of the luciferin and luciferase concentration.

According to a recent analysis with partially purified luciferin and luciferase (47), the nonluminescent oxidation of luciferin has an apparent activation energy of about 25,000 cal., while the luminescent oxidation has one of about 5000 cal. Under the conditions employed, there appeared to be an optimum temperature at about 23°C. for the latter reaction. At higher temperatures, the apparent velocity constant declined in a manner indicating an inactivation of the enzyme, by a reaction proceeding with about 50,000 cal., which is within the range typical of protein denaturations. In crude extracts and in the normal luminescent excretion of *Cypridina* the light may be extinguished by heating to as high as 65° and 54°C., respectively, but returns on cooling (90) showing that the heat inactivation is reversible if the reaction mixture is not heated too high or kept at high temperatures too long. A similar phenomenon occurs in bacteria, and with these organisms the diminution in luminescence at above optimum temperatures to a large extent is reversible by hydrostatic pressure, as discussed on page 235. It has not been determined whether pressure will reverse the heat extinction of luminescence in extracts, but it is reasonable to believe that it will, by analogy with the effects of pressure

with respect to bacterial luminescence. Apart from bacteria, the only report of pressure effects on bioluminescence appears to be that of Dubois and Regnard in 1884 (60). They subjected a lampyrid in water to six hundred atmospheres for ten minutes, after which it was still luminescent. Observations of its luminescence while under pressure, however, were evidently not possible with the apparatus they used.

In addition to the factors discussed above which modify the course of the reaction, chemical agents may retard or inhibit light production in at

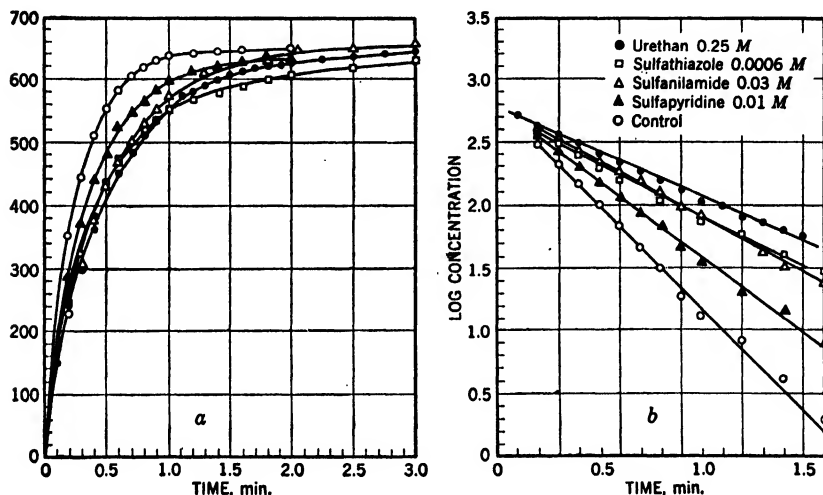


Fig. 4. Luminescent reaction of highly purified luciferin and partially purified luciferase of *Cypridina*, plotted as the integrated total light produced (in millivolts, see a) and as a first-order reaction (b).

least five distinct ways: (a) by combining with a critical group on the luciferin molecule, *e.g.*, the irreversible reaction between cyanide and purified luciferin, already referred to, whereby the total luminescence is reduced; (b) by quenching, in a reaction involving certain ions, whose mechanism is not understood (this also reduces the total luminescence); (c) by a reversible combination with one of the components of the system, independently of the denaturation of the protein, *e.g.*, sulfanilamide; (d) by a combination with agents, such as urethan, which promote a reversible denaturation of the protein; and (e) by substances which irreversibly denature the enzyme or destroy the luciferin. The action of azide (42) probably belongs in the second of these categories. The distinction between (c)

and (d) requires a temperature, and when possible also a pressure analysis, which have not been carried out with the extracted system. Figure 4 illustrates the retardation of the luminescent reaction by urethan and sulfa drugs without reduction of the total luminescence (123).

#### D. TOTAL LUMINESCENCE

In a given reaction, when correction is made for the spontaneous oxidation of luciferin, the total light is proportional to the amount of luciferin initially present, and is practically independent of the amount of luciferase in dilutions between 1 and 125 (3). Per unit of luciferin the total light is influenced by both the buffer system and the pH: it is less in phthalate than phosphate buffer, and less at pH 6.8 and 7.8 than at pH 6.0. It decreases from 2 to 3.5% per degree with rise in temperature between 16° and 28°C. More extensive data are needed in order to interpret these effects.

The effects of specific ions on the total luminescence is particularly interesting though not clearly understood. With the salts practically eliminated from the control solutions by dialysis and purification procedures, addition of the following, listed in order of their potency, caused increases of from 10 to over 100% in total light (6): NaCl, KCl, KBr, NaBr, KF, KNO<sub>3</sub>, K<sub>2</sub>C<sub>2</sub>O<sub>4</sub>, and K<sub>2</sub>SO<sub>4</sub>. A significant increase was caused by as little as 0.00048 *M* sodium chloride. A large decrease was caused by potassium thiocyanate and by potassium iodide which also reduced the velocity of the reaction. As in fluorescence, the negative ion evidently exerts the major influence. Iodide, thiocyanate, bromide, and chloride, which have the greatest effect in quenching fluorescence, also have the greatest effect on total luminescence, although the latter phenomenon is decreased by the first two and increased by the last two. The quenching effects of potassium iodide and potassium thiocyanate on luminescence may be largely overcome by the addition of sodium chloride (6).

### III. Mechanism of Bacterial Luminescence

#### A. SPECTROSCOPIC EVIDENCE

It has long been known that bacterial luminescence is intimately associated with the cells (165), and there is yet no certain evidence that luminescence has been observed apart from the living bacteria. Various means of disrupting cell structure—cytolytic agents, osmolysis, autolysis, mechanical grinding, supersonic vibration—apparently destroy the capacity for luminescence (138), although the activity of certain enzyme systems,

including catalase (186) and possibly also the cytochrome oxidase system (138), may increase after cytolysis. Several interpretations are possible in regard to the loss of luminescence, including the release of degradation products which prevent light emission. It would be eminently worth while, therefore, to carry out procedures for the separation of specific respiratory enzymes, such as flavoproteins, especially in view of the luminescence obtained from thoroughly dialyzed, concentrated luciferase preparations of *Cypridina* (124). It is within the realm of theoretical possibility that visible luminescence would be emitted, under appropriate conditions, during the *in vitro* oxidation of purified flavoproteins from ordinarily nonluminous cells.

A careful study of the emission spectrum of two marine and one freshwater species of luminous bacteria, as well as of certain chemiluminescences and fluorescences, has been made by Eymers and van Schouwenburg (64). The spectral energy distribution was found to be the same in each species and independent of the age of the cells, temperature, pH, and salt concentration. Assuming a symmetrical broadening of fundamental frequencies, analysis of the data indicated a fundamental frequency of  $18,200\text{ cm.}^{-1}$  in the luminescence of bacteria, *Cypridina* extracts, dimethyldiacridylum nitrate oxidation, and in the fluorescence of substances from cultures of *Pseudomonas putida*. The fluorescent spectrum of lactoflavin also indicated a symmetrically broadened, fundamental frequency of  $18,200\text{ cm.}^{-1}$ . Furthermore, in *Cypridina* luminescence was found a second fundamental frequency of  $21,250\text{ cm.}^{-1}$ , corresponding to a wave length of  $4705\text{ \AA}$ , which is very close to the absorption peak at  $4650\text{ \AA}$  during the oxidation of purified luciferin (43). In bacteria, a second fundamental frequency occurs at  $20,400\text{ cm.}^{-1}$ , corresponding to  $4902\text{ \AA}$  (Fig. 5). In an analysis of the luminescence of bacteria from the light organ of a deep-sea fish, Takase (178) found fundamental frequencies of  $21,200$  and  $19,200\text{ cm.}^{-1}$ . In  $0.5\text{ M}$  cane sugar the maximum intensity occurred at  $5200\text{ \AA}$ , while in  $0.8\text{ M}$  sodium chloride it occurred at  $4700\text{ \AA}$ , but the fundamental frequencies remained the same.

The results of a recent investigation of the effects of radiation on bacterial luminescence have provided extremely interesting and significant data with respect to the system in these organisms and its relation to that of *Cypridina*. Earlier studies had not indicated that illumination by visible light causes any effect on the intensity of bacterial luminescence (97), although it was known to reduce the luminescence of *Cypridina* extracts (96), and the inactivation spectrum by ultraviolet radiation (22,76,77,80) had



not been analyzed, except to some extent with regard to *Cypridina* extracts (45). Van der Kerk (184) and Kluyver, van der Kerk, and van der Burg (137) have now shown that bacterial luminescence may be affected by illuminating the cells with both visible and ultraviolet radiation, the inactivation spectrum extending from 270 to 480  $m\mu$ , with two strong bands at 290 and 405–410  $m\mu$ , and with two secondary maxima at about 320 and

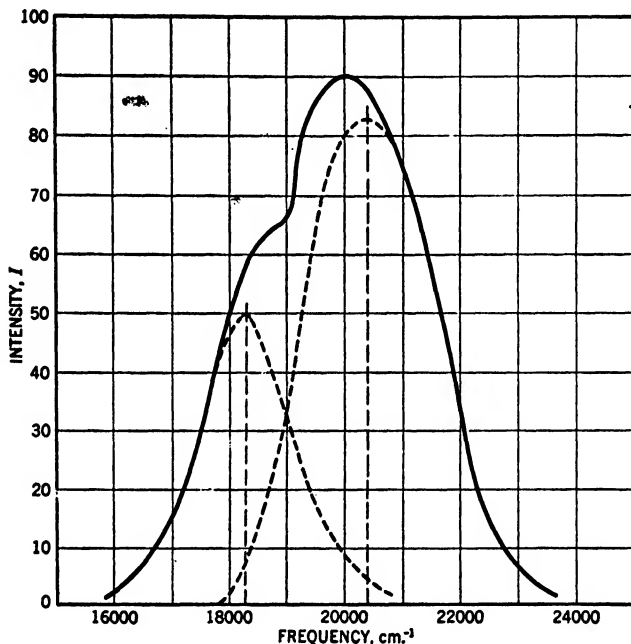


Fig. 5. Intensity-frequency curve of luminescence emitted by *Photobacterium phosphoreum* (64).

430  $m\mu$ . These results are in remarkable agreement with the absorption spectrum of purified *Cypridina* luciferin (43). The absorption at 465  $m\mu$  that appears during the oxidation of the latter evidently represents the oxidation product of luciferin. Consequently, this wave length would not necessarily be important in the inactivation spectrum. Although the inactivation of purified *Cypridina* luciferin by visible light appears to depend upon the presence of a sensitizer, it remains possible that the effects in bacteria occur in the luciferin directly, in addition to probable effects of the shorter ultraviolet wave lengths on the luciferase. On the basis of the

correspondence between the inactivation spectrum of bacterial luminescence, and the absorption spectrum of 1,4-naphthoquinone derivatives (69), as well as the evidence from the experiments of Chakravorty and Ballentine (39) and other facts, Kluver *et al.* suggest that dihydroluciferin is either identical with, or closely related to, 2-(hydroxyacetyl)-1,4-naphthoquinone, which would make it related to vitamin K derivatives, whose synthesis by bacteria has been demonstrated (53).

The spectroscopic data, together with the fact that bacterial luminescence is evidently under the control of an enzyme which is readily denatured by heat as well as by certain other agents, constitute substantial evidence for assuming a fundamentally similar luciferin-luciferase system in both bacteria and *Cypridina* extracts.

#### B. RELATION TO GENERAL RESPIRATION

Extensive observations and experiments on "shining wood" and "shining fish" were made by Robert Boyle, who showed in 1667 that bioluminescence may be reversibly extinguished by exhausting and readmitting air (24). Modern studies have demonstrated that almost incredibly small concentrations of molecular oxygen are sufficient to give rise to visible luminescence and there is only one well-authenticated case of bioluminescence under conditions rigidly excluding molecular oxygen (103). Most recently, a careful study by Meyer (156) has indicated that bacterial luminescence remains visible at a concentration of  $10^{-10}$  part of oxygen in nitrogen. At tensions between about 0.14 and 100% oxygen, luminescence remains at a maximum (173). As the partial pressure of oxygen is decreased, the rate of respiration begins to decrease sooner than the intensity of the luminescence (174,185). It is apparent that, in luminous bacteria, a fraction of the total oxygen consumption of the respiring cells is used, probably directly, in the luminescent reaction. Additional evidence that this is the case has been provided by the recent studies of van der Kerk (184). It is possible that a much larger fraction may be accounted for in the dark oxidation of the luminescent system, and it is very difficult to arrive at quantitative figures concerning the relative amounts of oxygen that go into (a) the actual luminescent oxidation, (b) the non-light-emitting oxidation of the same system, and (c) other systems involved in the total respiration. Studies on the action of inhibitors have provided information with regard to this problem, as well as the general relationship of the luminescent system to other pathways of hydrogen transfer in the general metabolism.

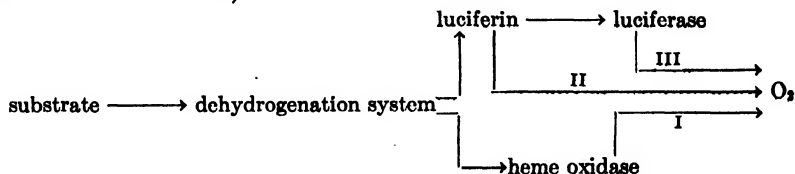
Bacterial luminescence is somewhat resistant to the action of cyanide (51,52,66,91,180,185), although relatively large concentrations reversibly inhibit luminescence and oxygen consumption proportionately (66,185). Much smaller concentrations greatly reduce oxygen consumption, showing that only a small amount of the total respiratory oxygen is required for the luminescent reaction. Moreover, by the addition of urethan or certain other inhibitors the intensity of luminescence may be greatly lessened, reversibly, without a large decrease in the rate of oxygen consumption (13,52,89,122,131,181,182,185). In an extensive study of the relation between the effects of cyanide, urethan, and oxygen tension, both singly and in relation to each other, on light intensity and total oxygen consumption, van Schouwenburg concluded that the action of cyanide is on the luciferin, while that of urethan is on the luciferase. The results of a study (133) on the "flash" of luminescence, which momentarily follows the admission of oxygen to cells that have been under partial or complete anaerobiosis, gave no evidence of a direct effect of cyanide. The effects of temperature, barbital, dinitrophenol and osmotic pressure have also been studied on this flash (172). Although further studies of the cyanide inhibition of bacterial luminescence have not been carried out, there is now substantial evidence, discussed in later paragraphs, that urethan acts on the protein of the enzyme. It is worth noting in passing that the cyanide inhibition of luminescence is unusual in that it is strongly related to the partial pressure of oxygen, much higher concentrations of the inhibitor being required for the same percentage of inhibition at low than at high oxygen tensions.

Azide inhibits bacterial luminescence (116) but its effects have not been studied extensively. The influence of carbon monoxide has been studied (51,175) thoroughly by van Schouwenburg and van der Burg (187), who find that both total oxygen consumption and luminescence intensity are affected, though not to the same extent, and that the relationships are somewhat complicated. Luminescence increases under gas mixtures with a low ratio of  $\text{CO}/\text{O}_2$ , and decreases when the ratio is high. Respiration is not increased, but undergoes an inhibition that is greater with higher  $\text{CO}/\text{O}_2$  ratios, and increasingly greater when the partial pressure of both gases, at a given ratio, is reduced by nitrogen. Strong illumination partially counteracts the action of carbon monoxide on respiration, and opposes both the stimulating and inhibitory effects on luminescence (187).

Luminescence is more sensitive than respiration to small concentrations of mercuric chloride. The amount of inhibition by this agent depends upon the concentration of the cells as well as that of the inhibitor,

and is not reversible on dilution, although there is evidence that it is reversible by hydrogen sulfide (109). The inhibition becomes greater with rise in temperature, but this effect is not reversible on cooling. In general, the action appears to resemble that of other protein denaturants, discussed later (pages 237–240), although in contrast to mercuric chloride, in many cases the other agents cause inhibitions that are easily reversible by dilution or cooling.

Further evidence concerning the relation of luminescence to the general pathway of hydrogen transfer derives chiefly from two sources; first, the effects of adding hydrogen donors to suspensions deficient in oxidizable substrates, especially washed cell suspensions in buffered salt solution, and second, the free energy change of luminescence. With regard to the latter, it may be said at once that the wave length of maximum luminescence, 4750 Å, represents a free energy change of 60,700 cal. which corresponds closely with the 57,340 cal. average free energy of two hydrogen atoms in the oxidation of glucose (124). As for the former, it is significant that luminescence, and also oxygen consumption, immediately undergo a marked increase if glucose, or certain other sugars, is added to washed cell suspensions (111–114,117). Luminescence may then remain at a maximum for appreciably long intervals, half an hour, at optimum pH and temperature, but then decreases approximately logarithmically over a period of several hours during which reproduction of the cells is essentially prevented by the absence of a nitrogenous substrate (114). Peptone, which usually contains appreciable concentrations of glucose, also increases the brightness of luminescence in cell suspensions (185) in too short a time for its effect to be accounted for in terms of an increase in number of cells. Thus, it is clear that a part of the substrate hydrogen is transferred by way of the light-emitting system and furnishes the energy for luminescence. On the basis of this and other facts, van Schouwenburg (185) proposed the first well-defined "scheme" for the place of luminescence in aerobic bacterial metabolism, as follows (arrows indicate the direction of transfer of hydrogen atoms or electrons):



There seems to be no reason to doubt that this scheme is fundamentally

correct. Nakamura (159-162) has suggested relationships between the luminescent and certain other catalytic systems, though the evidence is not altogether clear. His hypothesis—that luminous bacteria lack catalase, the hydrogen peroxide formed in respiration being eliminated by directly oxidizing (with light emission) the bacterial luciferin (159)—is somewhat in error, inasmuch as the presence of abundant catalase in these organisms

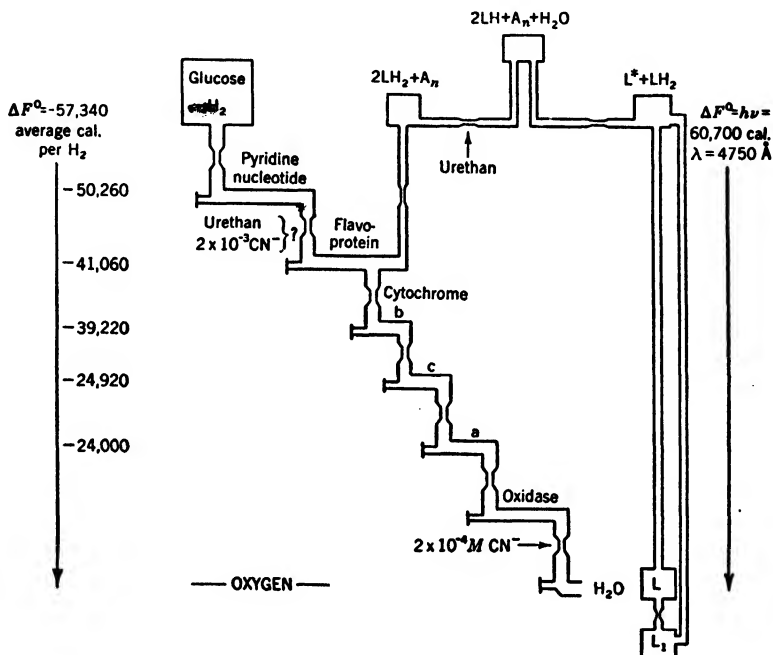


Fig. 6. Diagram representing the relation between the luminescent system of bacteria and the general pathway of hydrogen or electron transfer in respiration (10,126). The drawing is not to scale.

has been demonstrated (186). The most recent scheme, consistent with the established facts concerning luminescence in both bacteria and the extracts of *Cypridina*, as well as with the earlier, less detailed scheme of van Schouwenburg, is that of Johnson, Eyring, and collaborators (Fig. 6).

In the figure, the free energy which each system liberates is indicated as two hydrogens, or two electrons plus two protons, which are liberated to form water. Constricted tubes indicate the chemical inertia of processes which may be much further inhibited by agents such as urethan or cyanide, indicated opposite the sensitive pathway. Presum-

ably all the hydrogen or electron acceptors may enter into a variety of side reactions suggested by the closed-off pathways to the left. The flavoprotein, in spite of its energy's being lower than  $\text{LH}_2$ , will serve as a hydrogen-transfer catalyst to  $\text{LH}_2$ , providing the hydrogen atoms on the flavoprotein are not drawn off too rapidly by the cytochromes or side reactions. The proportionate inhibition of oxygen consumption and luminescence by the higher concentration of cyanide (185) indicates action on an intermediate common to both processes. In the scheme, this intermediate is taken to be flavoprotein.

It is necessary to postulate at least two autoxidizable enzymes. This is in accord with the view that has been held by Harvey and co-workers, on the basis of differences in the temperature-activity curves of luminescence and total oxygen consumption, as well as differential effects of inhibitors and other factors (170,171,175,180).

#### IV. Physical Chemistry of the Control of the Luminescent Reaction

##### A. RATE-CONTROLLING REACTIONS

Kinetic studies during the past five years have considerably clarified the mechanisms controlling the intensity of bacterial luminescence, and have made possible a rational interpretation of some general phenomena in the effects of such factors as temperature, hydrostatic pressure, and inhibitors on enzyme reactions. The chief aspects of these studies (33,68,118, 119,123,125-127,132,143) are summarized briefly in the following paragraphs.

The general procedure has been cultivation of the bacteria on agar plates and suspension of the organisms in an approximately isotonic salt solution containing sodium chloride and phosphate buffer. To this is added glucose in a final concentration of about 0.01 *M*, and a stream of oxygen or air is bubbled through. Under these conditions the intensity of luminescence is essentially constant for half an hour at room temperature, or much longer if kept at 3° to 5°C., and may be readily measured by a photoelectric cell or various other methods. The constancy of luminescence indicates that the luminescent reactions are proceeding at a maximum under the conditions imposed and that the concentration of luciferin as well as luciferase may be considered constant. Glucose evidently increases luminescence by increasing the amount of  $\text{XH}_2$  in reaction (1) (page 222), thus making more luciferin available. Hydrogen ions may also be an important factor limiting the over-all rate, and hence the intensity of luminescence, by opposing the dissociation of  $\text{ALH}$  in reaction (4); similarly, hydroxyl ions may be limiting, by removing the H on the  $\text{LH}$ . The net rate

of the over-all reaction is obviously influenced by a number of equilibrium and rate constants, but in the scheme described it is evident, from the experiments with *Cypridina* extracts, that reaction (2) is sufficiently slower than those which follow to be of predominating importance. The general expression for the intensity of luminescence,  $I_1$ , is then given by equation (1), in which  $b$  is a proportionality constant,  $k_1$  the rate constant,  $\text{LH}_2$  represents luciferin, and  $A_n$  the active form of the enzyme:

$$I_1 = bk_1(\text{LH}_2)(A_n) \quad (1)$$

The rate constant is defined by equation (2), according to the Theory of Absolute Reaction Rates (67,83). In equation (2),  $k'$  refers to the specific reaction rate,  $K^\ddagger$  the equilibrium between the normal and activated states of the reactants,  $\Delta F^\ddagger$  the free energy,  $\Delta H^\ddagger$  the heat,  $\Delta E^\ddagger$  the energy,  $\Delta V^\ddagger$  the volume change, and  $\Delta S^\ddagger$  the entropy, all of activation,  $p$  the hydrostatic pressure,  $T$  the absolute temperature, and  $R$  the gas constant. The expression  $\kappa(kT/h)$  is the universal frequency for the decomposition of the activated complex in all chemical reactions. In this,  $\kappa$  is the transmission coefficient, usually equal to 1,  $T$  the absolute temperature,  $k$  the Boltzmann constant, and  $h$  Planck's constant.

$$k' = \kappa \frac{kT}{h} K^\ddagger = \kappa \frac{kT}{h} e^{-\Delta F^\ddagger/RT} = \kappa \frac{kT}{h} e^{-\Delta H^\ddagger/RT} e^{\Delta S^\ddagger/R} = \kappa \frac{kT}{h} e^{-\Delta E^\ddagger/RT} e^{-p\Delta V^\ddagger/RT} e^{\Delta S^\ddagger/R} \quad (2)$$

In equation (2) the double dagger has the meaning "of activation," rather than "of reaction" appropriate to the corresponding equation (3) for equilibria:

$$K = e^{-\Delta F/RT} = e^{-\Delta H/RT} e^{\Delta S/R} = e^{-\Delta E/RT} e^{-p\Delta V/RT} e^{\Delta S/R} \quad (3)$$

#### B. ACTION OF TEMPERATURE, HYDROSTATIC PRESSURE, AND INHIBITORS

From equations (1) and (2) it is apparent that, with the concentration of  $\text{LH}_2$  and of  $A_n$  essentially constant, the intensity of the light will vary with the temperature and hydrostatic pressure according to the activation energy,  $\Delta H^\ddagger$  (indistinguishable from the " $\mu$ " of the Arrhenius equation at constant pressure), and the volume change of activation,  $\Delta V^\ddagger$ . It is only recently that a thorough study with hydrostatic pressure has yielded information about the latter of these constants. As for the former, however, it has been known for some time that, throughout a temperate range well below the temperature of maximum luminescence, or the "optimum," the

change in intensity with temperature conforms to the Arrhenius relation (158,170) with apparent activation energies of 27,000 and 22,400 cal. for a "fresh-water" and a marine species, respectively, under the conditions employed. A rather similar curve relating luminescence intensity and temperature has been found in experiments with bacteria from deep-sea fish (1). In approaching the optimum the straight-line relation between the logarithm of light intensity and the reciprocal of the absolute temperature deviates in the manner familiarly encountered with enzyme reactions, the slope decreasing and then changing signs at the optimum, beyond which the enzyme is rapidly inactivated with further rise in temperature.

An observation of crucial importance in the interpretation of the intensity-temperature relationship is that the diminution of luminescence caused by temperatures above the optimum is readily reversible on cooling, provided that the system is not held at high temperatures sufficiently long for irreversible destruction to take place to a considerable extent. This phenomenon was reported as long ago as 1892 by Eijkman (63), by Tollhausen (183) in 1889, and by Tarchanoff (179) in 1901. It was also observed by Beijerinck (22) and by Harvey (88). Its significance was not appreciated, however, until the pressure-temperature studies of Johnson, Brown, and Marsland in 1942, who independently rediscovered the reversible heat inactivation, and at the same time showed that, at the high temperatures, hydrostatic pressures of some 500 atmospheres also reversibly counteracted the diminution of luminescence, as well as the inhibitory effects of certain lipide-soluble narcotics at the optimum temperature. These pressure effects were in contrast to the action of increased pressure at low temperatures in the absence of narcotics, where pressure caused a logarithmic decrease in intensity, which was also reversible when the pressure was released. The quantitative data were analyzed in collaboration with Eyring and Magee, and the results have formed the basis of the further investigations and rational interpretations.

The high temperature coefficient of the reversible heat inactivation indicates that the phenomenon is due to a reversible denaturation of a protein, as has been described for proteolytic enzymes by Kunitz and Northrop (140) and Anson and Mirsky (8). The large pressure effects are also indicative of a very large molecule. The quantitative relation between temperature and luminescence intensity at different pressures can be described satisfactorily (Fig. 7) on the simple theory that the intensity increases with rise in temperature in proportion to the rate of the reaction, in accordance with equation (1), while at the same time it decreases in propor-



tion to the reversible denaturation of the enzyme by an equilibrium reaction, with constant  $K_1$ . By taking the latter into account, equation (1) becomes:

$$I_1 = \frac{bk_1(LH_2)(A_o)}{1 + K_1} \quad (4)$$

in which  $(A_o)$  represents the total amount of native, active enzyme,  $(A_n)$ , plus the reversibly denatured, inactive form,  $(A_d)$ . Since the actual con-

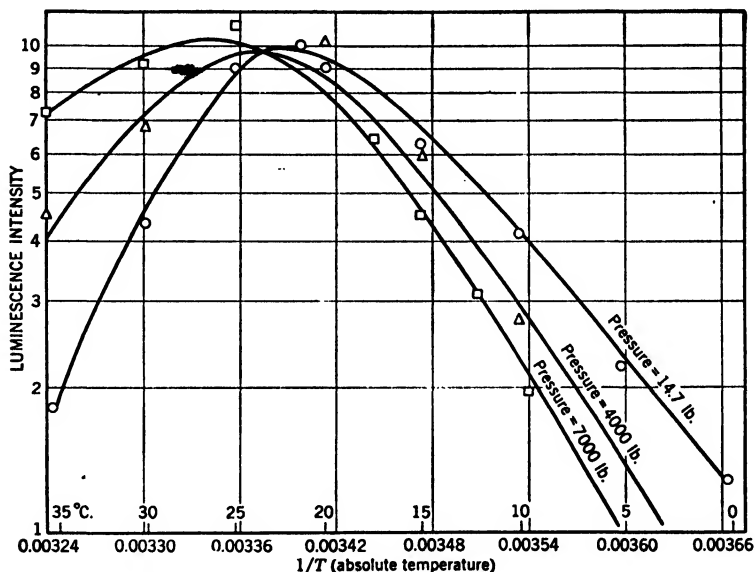


Fig. 7. Relation between luminescence intensity and temperature at normal pressure and at increased hydrostatic pressures of 4000 and 7000 lb. per sq. in., respectively, with *P. phosphoreum*.

centration of the reactants in the living cell cannot be readily determined, the numerator contains unknown terms,  $(LH_2)$ ,  $(A_o)$ , and  $\Delta S^\ddagger$ , appropriate to rate constant  $k_1$ . The apparent activation energy, however, can be obtained from experiments, and the other terms in the numerator may be considered constant under given experimental conditions. Thus, the unknown constants may be included in a single constant,  $c$ , and the equation for luminescence then becomes:

$$I_1 = \frac{cTe^{-\Delta E^\ddagger/RT} e^{-p\Delta V^\ddagger/RT}}{1 + e^{-\Delta E/RT} e^{-p\Delta V/RT} e^{\Delta S/R}} \quad (5)$$

which describes fairly closely the quantitative variations in intensity through the range of reversible effects of both temperature and pressure at neutral pH.

In Figure 7, circles, triangles, and squares represent experimental data (33). The smooth curves were calculated (68) in accordance with equation (5), but with recognition of a temperature dependence of  $\Delta V$  and of  $\Delta V^\ddagger$ . The numerical values of the rate and equilibrium constants used were as follows:

$$\begin{aligned}\Delta E^\ddagger &= 17,220 \text{ cal.}; \quad \Delta V^\ddagger = 546.4 - 1.813 T, \text{ in cc.}; \\ \Delta E &= 55,260 \text{ cal.}; \quad \Delta S = 184 \text{ entropy units}; \quad \text{and } \Delta V = -922.8 + 3.206 T, \text{ in cc.}\end{aligned}$$

The temperature relationships of luminescence indicate an apparent activation energy of between 15,000 and 30,000 cal. for  $k_1$  in different species, and a heat of reaction of about 50,000 to 90,000 cal. in the denaturation equilibrium,  $K_1$ . The pressure relationships indicate that the enzyme reaction proceeds with a large volume increase of activation, on the order of 50 cc. per mole, while the denaturation occurs with a large volume increase of reaction, between 60 and 120 cc. per mole. In the analyses by Eyring and Magee a temperature dependence of the values of  $\Delta V$  and  $\Delta V^\ddagger$  is assumed, which conforms more closely to the data (Fig. 7), and indicates that the reactions are somewhat more complicated than the simplest theory takes into account, although the complications are minor in comparison with the chief effects.

Equation (4) is of fundamental importance in analyzing the physical chemistry of inhibitors which form equilibrium combinations with the enzyme in a manner that affects its catalytic activity. For, it is apparent that such combinations may be either with bonds that are not involved in the reversible denaturation (type I), or with bonds that are (type II). Different effects of temperature and of pressure would be anticipated in the two cases. At a given concentration, the percentage of inhibition by a type I substance might be expected to decrease with rise in temperature, as the enzyme-inhibitor complex is dissociated, and the pressure effect might be expected to be small. On the other hand, the type II substance combines in a manner that promotes the denaturation, so that a rise in temperature would be expected to increase the percentage of inhibition, and, since the equilibrium change involves a large  $\Delta V$ , the effects of pressure might be expected to be pronounced. These relationships have been abundantly demonstrated, sulfanilamide acting as type I, and urethan, alcohols, ether, and substances of a similar nature as type II. The general equation follows for luminescence intensity in the presence of added in-

hibitors of both types when the inhibitors do not form complexes with each other as well as with the enzyme:

$$I_2 = \frac{bk_1(LH_2)(A_0)}{1 + K_1 + K_2X^r + K_1K_3U^s} \quad (6)$$

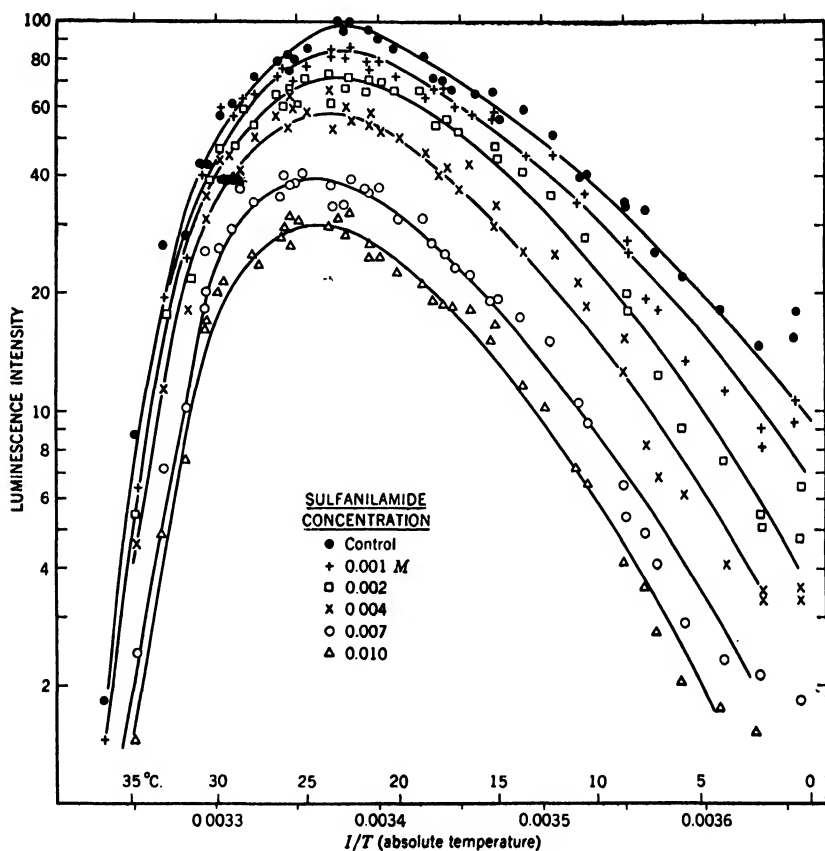


Fig. 8. Relation between luminescence intensity of *P. phosphoreum* and temperature, with corresponding portions of a given cell suspension containing various concentrations of sulfanilamide (126). Luminescence intensity is plotted on the logarithmic scale of the ordinate.

in which  $K_2$  represents the equilibrium constant for the combination of the enzyme with  $r$  molecules of (X), a type I inhibitor, and  $K_3$  that with  $s$  molecules of (U), a type II inhibitor. In regard to the latter, it should

be noted that there is no physical way of distinguishing whether the inhibitor combines with the denatured form,  $A_d$ , according to the constant,  $K_3$ , or with the native form,  $A_n$ , according to the constant,  $K_1K_3$ .

Analytical formulations appropriate to two respective types of inhibitors may be arrived at by dividing equation (4) by equation (6), thereby eliminating the terms in the numerator, and then simplifying. The results are given in equations (7) and (8) for types I and II, respectively. Agreement between data from experiments and the

$$\left(\frac{I_1}{I_2} - 1\right) = K_2 X \quad (7)$$

$$\left(\frac{I_1}{I_2} - 1\right) \left(1 + \frac{1}{K_1}\right) = K_1 U \quad (8)$$

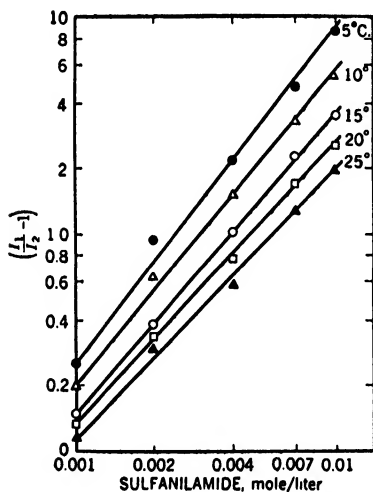


Fig. 9. Analysis of data in Figure 8 according to equation (7). The slopes of the lines are slightly more than 1.

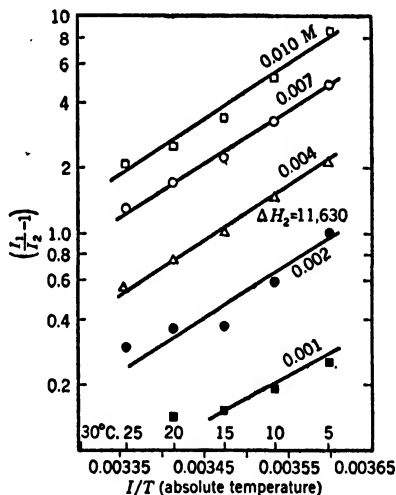


Fig. 10. Analysis of Fig. 8 data according to eq. (7), for heat of reaction in the equilibrium between sulfanilamide and the luminescent system.

theory assumed is tested by plotting the logarithm of the expression on the left successively against first, the logarithm of the molar concentration of inhibitor, the temperature and pressure remaining constant; second, the reciprocal of the absolute temperature, with concentration of inhibitor and the pressure constant; and third, the pressure, with temperature and concentration of inhibitor constant. A straight line relation should result in each case, unless the mechanism is more complicated than the theory takes into account. From the slopes of the lines in these plots we obtain: first, the values for  $r$  or  $s$ , the ratio of inhibitor to enzyme molecules in the equilibrium concerned;

second, the value of  $\Delta H$ , the heat of reaction; and third, the value of  $\Delta V$ , the volume change of reaction. With the aid of these constants it is then possible to calculate with some success the intensity of luminescence at various temperatures, pressures, and concentrations of the substance concerned. In luminescence, sulfanilamide acts as a type I inhibitor, and is not appreciably affected by pressure. The analysis of its action is illustrated in Figures 8, 9, and 10. Alcohol, on the other hand, acts largely as a type II inhibitor, and its effects depend on pressure (Fig. 11) as well as temperature.

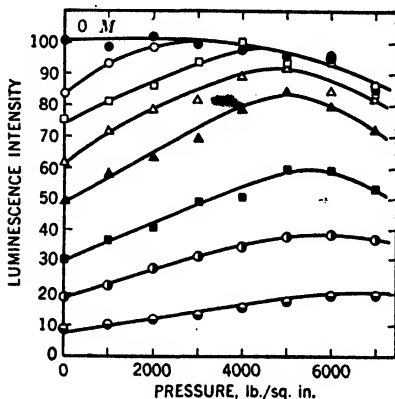


Fig. 11. Relation between luminescence intensity of *P. phosphoreum* and hydrostatic pressure, at 17.5°C. and neutral pH, in corresponding portions of a given cell suspension to which was added ethyl alcohol in final molar concentrations (in order, from uppermost to lowest curve, respectively) of 0, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, and 1.5 (126).

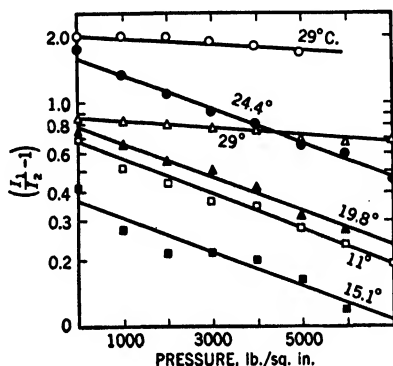


Fig. 12. Influence of hydrostatic pressure on the inhibition of luminescence of *P. phosphoreum* by 0.5 *M* ethanol at different temperatures (126). Duplicate lines for 29°C: are from repeated experiments with 2 different cell suspensions. The lines are parallel, though the per cent inhibition was not the same in the 2 experiments.

The results of a pressure analysis at different temperatures are illustrated in Figure 12, where the amount of inhibition increases with the expression,  $(I_1/I_2 - 1)$ , along the ordinate. The results are from several experiments, including two at 29°C. The slope of the line at 24.4°, which is practically parallel to those for lower temperatures, indicates a volume increase of reaction of 62.6 cc. per mole.

The fact that the pressure effect on the alcohol inhibition tends to disappear at the higher temperatures indicates that the volume change in the equilibrium combination between the alcohol and the enzyme protein is very small, the pressure effects at lower temperatures being mediated through the ordinary temperature- and pressure-sensitive denaturation equilibrium.

The effects of hydrogen and hydroxyl ions, throughout the range of concentrations which do not denature the protein of the enzyme, conform to the type I inhibition, and may be analyzed by the formulation of equation (7). The results depend upon the buffer system employed, and its concentration, showing that the companion ions also play a part. At the optimum temperature, and in 0.125 *M* phosphate buffer in 0.25 *M* sodium

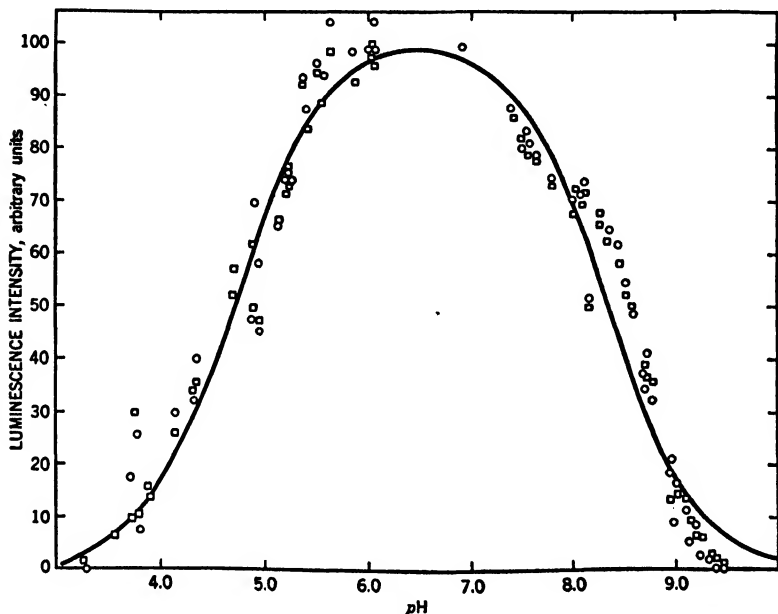


Fig. 13. The pH-activity curve for luminescence of *P. phosphoreum* at optimum temperature in phosphate-buffered sodium chloride solution to which was added small amounts of hydrochloric acid or sodium hydroxide to give the various pH values.

chloride, the pH-activity curve of luminescence of *Photobacterium phosphoreum* is illustrated in Figure 13, in which the smooth curve was calculated according to equation (9); the points are from experiments (126).

$$\left(\frac{I_1}{I_2} - 1\right) = K_1[H^+] + K_2[OH^-] = 4.84 \times 10^4[H^+] + 4.8 \times 10^4[OH^-] \quad (9)$$

The concentration analyses indicate one hydrogen and one hydroxyl ion, respectively, in the equilibrium combination, and their effects may be accounted for on the basis of reactions (4) and (5) (page 222). A rise in

temperature decreases the percentage of inhibition caused by an acid  $pH$ , in comparison with a neutral  $pH$ —an effect which may be attributed to the dissociation of ALH in reaction (4), for which the analysis indicated a heat of reaction of about 15,000 cal., a value very close to previous determinations of the apparent activation energy at neutral  $pH$ . It is also significantly close to the value found for the equilibrium with sulfanilamide. The various relationships referred to in this paragraph have been discussed in some detail (126).

It is perhaps worth pointing out explicitly the enormous changes that occur in the apparent activation energy of luminescence, and apparent heat of the denaturation equilibrium under the influence of various factors. For example, by changing the  $pH$  from approximately 7 to 5, the apparent activation energy increases by as much as 20,000 cal., and the addition of sulfanilamide may cause increases of a similar magnitude. The denaturation equilibrium, however, is hardly affected by these agents (except, perhaps, in the range of high temperatures and concentrations), with the result that the apparent optimum temperature shifts to a few degrees higher. Agents that promote the reversible denaturation, on the other hand, may greatly decrease the apparent activation energy, and lower the heat of denaturation, with the result that the observed optimum temperature occurs several degrees lower in their presence. These reversible changes in a given suspension of cells may be greater than the variations normally observed among different species under the same conditions for each.

### C. MIXTURES OF TWO INHIBITORS

There is evidence that type I and type II inhibitors tend to enter into a loose combination with each other. When the two are simultaneously present, the inhibition may become considerably less than that observed with one or the other separately, in the same concentrations. At low temperatures, for example, where moderate concentrations of urethan have only a slight effect, while sulfanilamide causes a much greater inhibition than at high temperatures, the addition of small concentrations of urethan markedly opposes the action of sulfanilamide, and the intensity of luminescence may increase several fold.

In this case, the urethan is considerably in excess in comparison with the concentration of sulfanilamide, and, from measurements of the intensity of luminescence in the presence of the two inhibitors separately and together, it is possible to arrive at a value for the equilibrium constant,  $K_4$ , in the equilibrium between the inhibitors, by:

$$\left[ \left( \frac{I_1 - I_{s_0}}{I_u - I_{s+u}} \right) \left( \frac{I_{s+u}}{I_{s_0}} \right) \right]^{1/r} - 1 = K_4(U_0) \quad (10)$$

in which the notation is as follows:  $I_1$ , luminescence intensity of the control without added inhibitor;  $I_{x_0}$ , intensity with sulfanilamide alone;  $I_u$ , intensity with urethan alone,  $I_x + u$ , intensity with both sulfanilamide, in initial concentration  $x_0$ , and urethan simultaneously present;  $U_0$ , molar concentration of urethan added;  $r$ , ratio of urethan

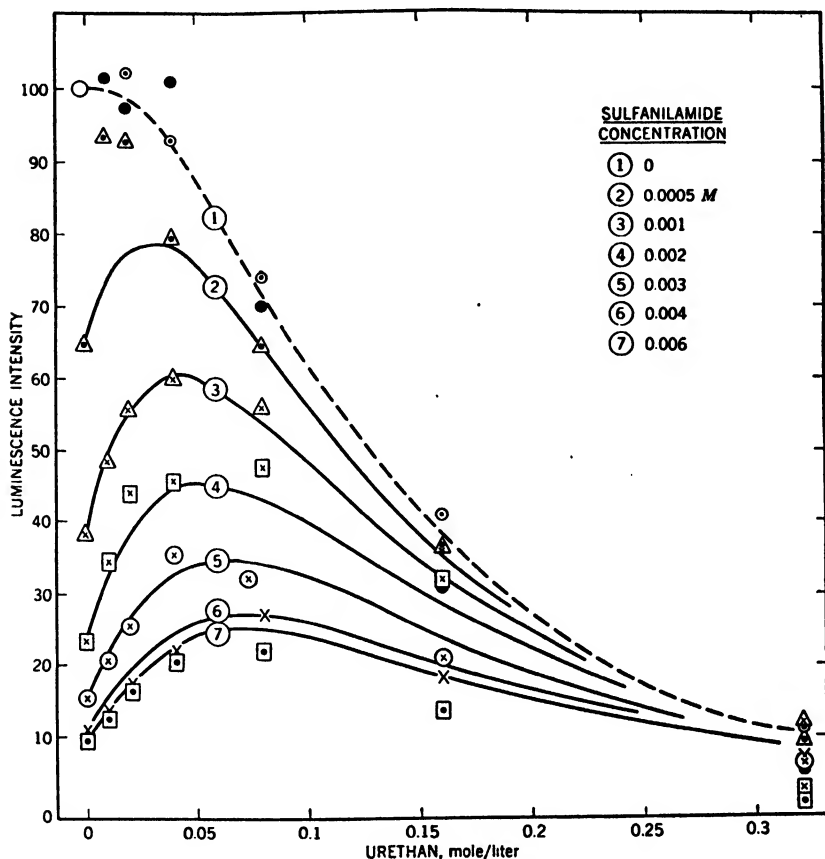


Fig. 14. Luminescence intensity of *P. phosphoreum* at 5°C. in relation to various concentrations of urethan alone (dotted line), sulfanilamide alone (points below 100, at urethan = 0), and mixtures of the two in various proportions and concentrations.

to sulfanilamide in the equilibrium whose constant is  $K_4$ . This formulation is extremely sensitive to experimental error. The average of a number of determinations of  $K_4$ , however, permits fairly close predictions of the intensity of luminescence in the presence of various combinations of sulfanilamide and urethan concentrations, except in high concentrations of the latter, where some irreversible effects take place, as well as in very



low concentrations of the same, where at low temperatures the inhibition of luminescence is less than expected (Fig. 14). In the figure, solid lines represent the curves calculated, with the aid of equation (10), from the effects of two inhibitors separately, assuming that when sulfanilamide and urethan are present simultaneously they form a complex with each other, in a molecular ratio of 1:1, with an equilibrium constant of 50. The points represent the data from experiments (125).

The same mechanism of complex formation possibly accounts for the antagonism by urethan of the sulfanilamide inhibition of bacterial growth, and for certain other "nonspecific" antagonisms (116,126,148). Evidence for complex formation apart from a biological system is apparent in the effects of urethan, ether, and other type II agents on the solubility of sulfanilamide in neutral solution (125).

Analytical formulations have been derived for mixtures of the same type of inhibitor, when one or neither is present in excess, and furnish the quantitative basis for measuring synergism and antagonism under the conditions specified. Unless one of the inhibitors is in considerable excess, the formulations become complicated and rather difficult to apply to data from experiments (125).

## V. Irreversible Denaturation of the Luminescent System

### A. RELATION TO TEMPERATURE AND HYDROSTATIC PRESSURE

The destruction of the luminescent system under the influence of various factors may be followed readily by measuring the decrease in luminescence intensity with time, of a washed cell suspension, in comparison with a control of uniform brightness under noninjurious conditions. Thus, at temperatures above the optimum, the intensity is immediately reduced, but on cooling at once returns to its former maximum. At the higher temperatures, however, the intensity decreases logarithmically with time; and after successively longer periods the luminescence of suspensions, when cooled to the optimum temperature, fails to return to the former maximum by very close to the amount that it has decreased during exposure to the higher temperature. When the logarithm of the rate of thermal destruction at various temperatures is plotted against the reciprocal of the absolute temperature, a straight line is obtained over a range of more than one hundred times in reaction rate, and with slope equivalent to an activation energy, in the species studied, of about 90,000 cal. This is about 20,000 cal. higher than the heat of reaction in the reversible denaturation of luminescence in this species under the same conditions, a fact which makes it possible to observe the latter reaction without permanently impairing the

system. The analysis of the data indicated that the two denaturations are separate, involving the breaking of different bonds. Factors which promote the one, however, will in general promote the other—for example, urethan, which, in lower concentrations at the higher temperatures, or in higher concentrations at the lower temperatures, causes a reversible denaturation similar to that brought about by alcohol, also promotes an irreversible destruction, which becomes apparent by raising the temperature or increasing the concentration. The logarithm of the velocity of this reaction against the logarithm of concentration gives a straight line whose slope is about 1.5, indicating an average ratio of one and one-half more molecules of urethan combined with the activated molecules undergoing irreversible destruction than the normal molecules.

The effects of urethan and other type II inhibitors are in contrast to the effects of type I inhibitors, which do not, in the cases investigated, cause irreversible effects. The latter produce inhibitions that are constant with time, whereas inhibitions by the former agents except in low concentrations or at low temperatures, tend to become greater with time. These observations, of course, have some general implications.

#### B. RELATION TO pH

The rate of the permanent denaturation may be greatly influenced by both pH and by pressure. It appears to be slowest at about pH 6.5 in 0.125 M phosphate buffer, possibly the isoelectric point, and increases as the reaction is made more acid or more alkaline, although at ordinary temperatures the system appears to be stable in the acid range down to pH 4.0. The effects of pressure at a neutral pH and constant temperature are illus-

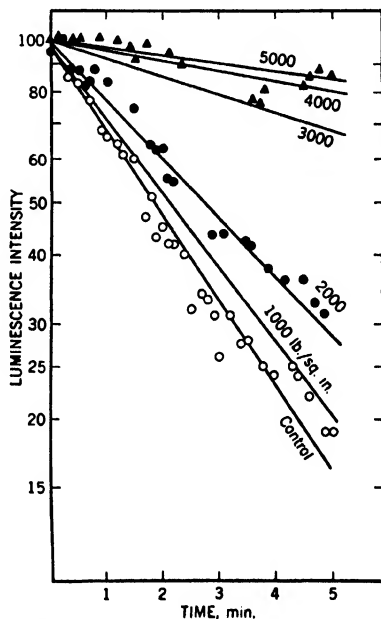


Fig. 15. Influence of hydrostatic pressure on rate of thermal destruction of the luminescent system of *P. phosphoreum*, at 34°C., about 14° above the optimum temperature of luminescence in this species (126).



luciferase is the molecule concerned, on the basis of correspondence between the effects of urethan, etc. on the extracted system of *Cypridina* and on bacterial luminescence, and on the basis of the action of pressure in abolishing the inhibition of bacterial luminescence by these agents. In any case, of course, the theory of action is the same, whether it applies to only one specific enzyme, or to more than one involved in the same process. The agreement of the data with the simple mechanism assumed indicates that complicating reactions are relatively unimportant. For this, as well as other reasons, bacterial luminescence has unique advantages in the study of the control of biological reaction rates in living organisms.

On the assumption that it is only the luminescence system that is affected, the various equilibria and rate processes which influence luminescence by the factors discussed are summarized in Figure 16.

Only  $A_n$  takes part in catalyzing the light-emitting reaction. It is pictured schematically as a protein consisting of a long chain of amino acids held together in a folded configuration by secondary bonds, indicated by the small black dots. The square at the left end of the coil represents the prosthetic group. The molecules of X combine in a manner that prevents the function of the prosthetic group, and independently of the reversible denaturation ( $K_1$ ). The ratio of molecules in this combination ( $K_2$ ) is 1:1. With rise of temperature,  $A_n$  undergoes a reversible denaturation ( $K_1$ ) with a large heat of reaction and volume increase which presumably involves a loosening of the secondary bonds and unfolding of the protein. Urethan promotes this reaction by combining with the bonds made available in the unfolding, and may be pictured as combining with only the denatured form ( $A_d$ ), according to an equilibrium constant  $K_3$ , or with the native form ( $A_n$ ) according to the equilibrium constant  $K_1K_3$ , resulting in the same product in either case (AU). Between two and four urethan molecules combine per protein molecule. Sulfanilamide may likewise combine with this product, according to the constants indicated.

In the irreversible destruction, the evidence indicates that the bonds that are broken are not the same as those involved in the reversible denaturation. Thus, the rate process of destruction may be the same in  $A_n$  and  $A_d$ . This reaction involves a large volume increase of activation, and is promoted by urethan and similar agents. Analysis of the data concerning urethan action indicates that an average of one and one-half more molecules of urethan are combined with the activated form of the protein undergoing destruction than with the normal form. By the Theory of Absolute Reaction Rates, the activated molecules in all cases decompose at the universal frequency,  $\kappa(kT/h)$ . The products of the reaction, indicated by Y, may be the same in each case. The double daggers in the figure have the significance "of activation," and are used with the diagram of the protein to indicate the activated state. The equilibrium and rate constants are defined at the left of the figure.

The equilibrium or rate constant is defined in each case by equations (3) or (2), respectively (see page 234). Under appropriate conditions a

value for each constant may be arrived at with the aid of the formulations referred to above. The formulation for the irreversible denaturation, where it occurs as a first-order rate process, is essentially the same as that for the reversible denaturation. With regard to type I inhibitors, it will be recalled that they act independently of the denaturation of the protein, as if by combining with the prosthetic group, or in a manner that interferes with the activity of this group, and that, according to the theory outlined, the luciferin may be regarded as the prosthetic group of the enzyme.

## VI. Generality of the Pressure-Temperature-Inhibitor Relations Illustrated in Luminescence

Although it is hardly desirable or possible to include in this review the numerous studies of the action of pressure, temperature, and inhibitors on diverse biological phenomena, some general remarks in connection with recent advances are perhaps worth while, especially in regard to the action of pressure. Prior to 1942, the possibility that, under appropriate conditions, proteins might undergo denaturation with a volume increase of reaction or of activation and, therefore, be opposed or counteracted by hydrostatic pressure was apparently completely unnoted. On the other hand, it was well known that high pressures, of the order of ten times those referred to in this paper, at ordinary temperatures bring about the denaturation of certain proteins, *e.g.*, egg albumin (25,84), meat proteins (16), serum globulin (26), and tobacco mosaic virus (79,142), kill microorganisms (14,50,108,141,169), and inactivate antitoxin (15), bacteriophage (19), several viruses (17,18), and enzymes (146,147,154).

A retardation in the rate of denaturation of a pure protein in solution, comparable to the pressure effect on the thermal destruction of the luminescent system, thus assumes particular interest. Qualitative observations have recently shown that this phenomenon takes place in solutions of highly purified egg albumin and serum globulin (120). A pressure of 10,000 lb. per sq. in. practically prevents the precipitation of the latter protein at 65°C. and *pH* near neutrality. Quantitative studies of the kinetics of this denaturation have not been completely analyzed, but the indications are that a volume increase of activation on the order of 100 cc. per mole is involved (121). Small concentrations of ethyl alcohol accelerate the precipitation under both normal and increased pressure, as shown in Figure 17; the denaturation proceeds with a large volume increase of activation, of the order of 100 cc. per mole, as in the destruction of the

luminescent system. Moreover, the inactivation of partially purified antibody is opposed by such pressures at the same temperature. The kinetics are somewhat complicated, but at the start of the reaction there appears to be a volume increase of activation of at least 39 cc. per mole (134).

In contrast to the lethal effects of high pressure on bacteria, recent studies have demonstrated that the rates of disinfection of *Escherichia coli*, at temperatures above 45°C., or at lower temperatures in the presence of quinine, may be greatly slowed by the application of a pressure of 5000 lb. per sq. in. (130).

With regard to the action of pressure on specific enzyme reactions *in vitro*, it would be anticipated from the results with luminescence that a dual effect would be encountered, depending upon the temperature of the experiment; *i.e.*, the over-all rate should very likely decrease under pressure at temperatures below the optimum, but increase at temperatures above the optimum because of the greater effect at these temperatures on the reversible denaturation of the protein than on the enzyme reaction itself. There is no way to predict, *a priori*, whether a given enzyme will exhibit a pressure-sensitive, reversible denaturation, or have its activity reduced, in the native state, by pressure. There is evidence in the literature, however, that pressure does, indeed, reversibly retard various enzyme reactions, both with the extracted systems (23) and in living cells as judged by the velocity of the total reaction, such as fermentation, photosynthesis (167), and other processes. Moreover, stimulating as well as retarding effects of pressure on the rate of a given process have been noted with respect to oxygen consumption (72,73), muscle contraction (28,29,31,35-38,61,62), and action potentials of nerve (85). In muscle contraction, the important relation of the pressure effects to temperature, as well as to the species of organism, was recognized by Brown (30). While it is likely that among di-

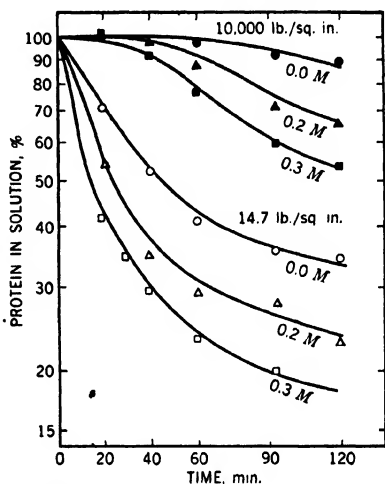


Fig. 17. Influence of hydrostatic pressure and of small concentrations of ethyl alcohol at both normal (lower three curves) and increased pressures on the rate of precipitation of highly purified human serum globulin at 65°C. (121).

verse, complex processes the effect of pressure will be mediated not only through an influence on the catalytic activity and specific reaction rates of enzymes, but also on other reactions, such as sol-gel changes, which likewise may involve large volume changes of activation or reaction (153), the available data suggest that the action of pressure on enzymes directly accounts for a significant part of the results observed. Unfortunately, sufficient data are generally lacking for a comprehensive analysis such as the one carried out with luminescence; and the complexity of many biological phenomena, such as muscle contraction, makes the analysis correspondingly more difficult. Recent studies on the growth rates of bacteria in relation to temperature, pressure, *pH*, and quinine, however, have given evidence of phenomena similar to those encountered in luminescence, in that : (a) the growth rate is reversibly diminished by temperatures above the normal optimum; (b) the temperature-activity curve for growth at normal pressure, throughout most of the range from low temperatures to those high enough to cause bacteriostasis, is quite satisfactorily described by the same equation as that for luminescence (Eq. 5); (c) pressures of 1000 lb. per sq. in. retard growth rates below the optimum temperature, but apparently cause slight increases in rates of growth at temperatures above the optimum; and (d) there is a reversal of the pressure effect, with increasing pressures, at intermediate temperatures. Higher pressures, *e.g.*, 5000 lb. per sq. in., retard growth at all temperatures, possibly through an influence on sol-gel changes during the process of cell division (130, 152), or an influence on the synthesis of new protoplasm.

There have been no reports thus far of experiments demonstrating whether or not the reversible diminution in catalytic activity of purified enzyme preparations, such as trypsin (8), is counteracted by increased hydrostatic pressure; but the pressure activation, under certain conditions, of myosin triphosphatase has been reported (32).

The relation between temperature and the quantitative effect of various inhibitors has not been a subject of intensive investigation for various reasons, for example: the process in question frequently appears too complex to lend itself to simple analysis; an understanding of the mechanism of the normal temperature-activity curve has been incomplete; and formulations for testing the conformity of experimental data to a clearly defined theory of action with reference to the reversible denaturation of proteins, in addition to more specific effects independent of this reaction, have only recently become available. Thus, efforts have been directed primarily at analyzing the relation between concentration and inhibition at a given tem-

perature, usually the "optimum." In this connection it should be pointed out that a useful formulation, the reciprocal of the expression,  $(I_1/I_2 - 1)$ , used in luminescence, has been derived by Fisher and collaborators (71), and applied to the effects of inhibitors on diverse processes at constant temperature and pressure (9,70,176).

Although data sufficiently extensive and accurate to justify analyzing on the basis of the theory and formulations for the temperature relationship of inhibitory action reviewed in this paper are very scarce, there are many suggestive observations that invite further study on both the relatively simple and more complex biological processes. For instance, the effect of atabrine on cytochrome reductase increases greatly with rise in temperature (86). Similarly, the potency of quinine in disinfection is markedly increased by a rise in temperature, and it has been shown that this effect is opposed by hydrostatic pressure (132). Instances are legion, of course, where the rate of disinfection by a given substance rises with temperature. Although the mechanism of drug action on excised tissues and in whole organisms is frequently complex, the reversible or irreversible denaturation of a critical protein is possibly concerned in phenomena of increased potency with rise in temperature, *e.g.*, propazone and diphenyloxazolidinedione on respiration of cerebral cortex slices (74,75), cymarín and coumíngine in frogs (48), diaminodiphenyl sulfone in mice (49), rotenone in goldfish (78), and other cases.

## VII. General Physiology of Luminous Bacteria

### A. RELATION BETWEEN GROWTH AND LUMINESCENCE

Luminescence is not a necessary accompaniment to growth inasmuch as cultures may develop in the absence of sufficient oxygen to give rise to visible luminescence. Dark variants are encountered under ordinary conditions of aerobic cultivation. Nonluminous strains have been produced by incubating certain species at above optimum temperatures (21). Heavy water diminishes both luminescence and respiration (105) and the cells treated with heavy water have been observed to grow subsequently without luminescing (192). Old cultures that have ceased to emit visible light sometimes develop small, secondary colonies on the surface of the older growth that are fairly brightly luminous (21,116).

An important factor in the brightness of a given strain and its variants is the pH change induced in the medium. The most generally employed medium contains 1% glycerol, which favors growth and luminescence, but



from which acid is gradually produced in amounts sufficient to extinguish the light and kill the organisms. If the acid is frequently neutralized by adding fresh alkali, or if a contaminating mold, *e.g.*, *Penicillium*, is present, luminescence and viability persist much longer (106). Thus, ordinary variations in the relative rates of acid and alkali production during growth would account in part for variations in brightness and duration of luminescence. The data of Giese (81) on the pH changes in a peptone-glycerol medium during the growth of dim and bright variants are in accord with this view. In view of the different effects of many substances on luminescence and oxygen consumption, there would seem to be no necessary correlation between the pH effects on luminescence, and the pH-activity curve for total respiration during the oxidation of various substrates, including peptone, glycerol, or organic acids such as maleic, malic, fumaric, succinic, and pyruvic. Giese found essentially similar pH-activity curves for the respiration of dim and bright variants with these substrates.

Semidark dissociants of bright strains sometimes respond to an addition of lactoflavin by an increase in luminescence (54).

The aerobic growth of cultures without visible luminescence may take place under various conditions. Nonluminous cultures of marine species grow abundantly if the salt concentration is reduced. The addition of appropriate concentrations of sulfanilamide may also result in nonluminous cultures (126). In either case, subcultures on the usual media exhibit normal luminescence. Growth without luminescence takes place in the presence of ether (149). Although there is no clear, general relationship between the action of substances on the luminescence of fully developed cultures, and on the growth of such cultures, some of the antibiotic compounds apparently affect the two processes in a quantitatively similar manner, thus making possible the use of bacterial luminescence as a quick assay for the potency of the growth inhibitor. This method has been used for aspergillic acid, but the correlation between inhibition of growth and of luminescence, respectively, is less distinct with various other antibiotic substances, and is absent in regard to pure penicillin which, under the conditions described, has no effect on luminescence (166). An assay for sulfonamides could be worked out on the basis of their effect on luminescence, but among various derivatives which affect this phenomenon not all appear to have chemotherapeutic significance (116).

The sensitivity of luminescence to many substances that cause harmful effects in animals invites preliminary tests for possible nontoxicity of new chemotherapeutic compounds. Unfortunately, the criterion is not

altogether reliable. For example, bacterial luminescence is only slightly affected by cyanide, except in relatively high concentrations.

#### B. GROWTH, RESPIRATION, LUMINESCENCE, AND FERMENTATION IN RELATION TO SALTS AND SUBSTRATES

The relation of various salts to growth, respiration, and luminescence of bacteria has been the subject of a number of studies, but the results have been conflicting and difficult to interpret. Osmotic pressure, as well as the specific ion, is an important factor, and the *pH* of the solution has not always been taken into account. In particular, it is not clear to what extent the luminescent system itself is directly affected. Complex changes in the whole organization of the cell take place in very hypotonic solutions; *e.g.*, when transferred from an isotonic medium to distilled water, marine luminous bacteria undergo an osmotic cytolysis accompanied by the loss of luminescence, oxygen consumption, motility, viability, and by the liberation of surface-active substances (107,128,129). The cells are apparently surrounded by a rather rigid membrane, which cracks without appreciable swelling, liberating part of the contents of the cell. Electron micrographs have shown some of the structural details associated with this phenomenon in several species (135).

Osmotic pressure is more important than a considerable concentration of salts for luminescence, growth, and respiration, inasmuch as these processes are not very greatly affected when the tonicity is maintained by sucrose instead of by salts (129). In solutions of increasing hypotonicity luminescence undergoes a diminution more readily than respiration, while the opposite is true in solutions of increasing hypertonicity. Viability is the last to be affected in either case.

Luminous cells may be repeatedly washed with isotonic (3%) sodium chloride with very little effect on luminescence, beyond that which is caused by the elimination of oxidizable substrates in the medium (89), showing that the sodium, potassium, and calcium balance of ions is not necessary. The results of some recent experiments, however, have indicated that magnesium is essential for the growth and development of luminous cultures (151), and that unfavorable concentrations of calcium can be partially antagonized by magnesium. It has been stated that both sodium and potassium are essential for luminescence in an amino acid medium (34) and that, while zinc and aluminum are inhibitory, their effects may be counteracted by calcium, strontium, and barium.

According to the observations of Claren (51) on the respiration of

washed cells, oxygen consumption is not greatly affected when the sodium is replaced by lithium, potassium, or ammonium, and the chloride by bromide and iodide. With glucose as substrate, magnesium greatly increased respiration in phosphate buffer, but had decidedly less effect, or was injurious, in various concentrations of sodium chloride.

Growth and luminescence of cultures take place with a large variety of salts to maintain tonicity. Molisch (157) found that *Bacterium phosphoreum* developed and luminesced in media containing 3% NaCl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, KI, KNO<sub>3</sub>, MgSO<sub>4</sub>, or K<sub>2</sub>SO<sub>4</sub>, but not MnSO<sub>4</sub>. With *Bacillus photogenus*, however, MnSO<sub>4</sub> was satisfactory. Practically no growth of either species occurred in the absence of salt. The results of other studies on the influence of various salts are not entirely in agreement with the above. For example, growth and luminescence do not always occur in potassium chloride (52,77,149), and isotonic calcium chloride is highly injurious to both the respiration and luminescence of mature cultures (129).

The nutritional requirements and utilization of various substances by luminous bacteria have been studied at length by Molisch (157), Beijerinck (20), de Coulon (52), and others. Recently, Doudoroff (55) studied the requirements of several species for growth and luminescence in an inorganic salt medium, to which was added as sole source of carbon amino acids or various simple organic compounds like glucose, glycerol, lactate, succinate, and fumarate. The requirements in general were simple. In one species, *P. phosphoreum*, methionine appeared to be an essential growth factor, and could not be replaced by any of a large number of amino acids, common growth factors, or other organic substances.

The respiration of washed cells of *Micrococcus cyanophos* was studied at some length by Claren (51), but measurements of luminescence were not included. The reversible retardation of respiration by high oxygen tensions, noted by Shoup (174) and van Schouwenburg (185), was studied with various oxidizable substrates, succinic, pyruvic, formic, fumaric, lactic, and *l*-malic acids, as well as with glucose, glycerol, and formaldehyde. The role of fumarate in oxidative metabolism was studied, including its hydrogenation by molecular hydrogen in the presence of these organisms. Observations were also made on the action of cyanide and narcotics (51).

McElroy (144) has investigated the action of inhibitors, including chloral hydrate, chloretone, barbiturates, and dinitrophenol, with respect to both oxygen consumption and luminescence of washed cells with glucose as substrate. The total respiration was increased by concentrations of inhibitors that greatly reduced luminescence. Chloral hydrate and chlore-

tone greatly increased the endogenous respiration. The effect of the inhibitors on both respiration and luminescence was to some extent dependent on whether glucose was present in detectable amounts or had been used up.

Analyses of the glucose fermentation products of several facultatively anaerobic species of luminous bacteria have been made by Doudoroff (56). The products included formic, acetic, lactic, and succinic acids, ethyl alcohol, carbon dioxide, and acetylmethylcarbinol. Of the species studied, only *P. phosphoreum* produced hydrogen and, occasionally, 2,3-butenediol.

### C. EFFECTS OF RADIATIONS

Ultraviolet light affects viability more readily than luminescence (22,77,80), and doses which just prevent reproduction affect respiration only after a lapse of several hours (80). Large doses cause an almost immediate, proportionate decline in respiration. Survivors of a population of cells, in which only 0.01% of the cells remained viable after radiation, developed normally and showed no greater resistance to ultraviolet than the controls.

The effects of visible radiations have been discussed on pages 227-229.

Negatively, but not positively, ionized air caused an increase in luminescence, while neither ion affected growth, according to the observations of Hin Lin Hsu (110). The effects of radium emanations and other radioactive substances have been studied by Omelianski (164), Beijerinck (22), Zirpolo (191), Voormolen (188), and Rerabek and Hykesova (168), and both increases and decreases in luminescence have been noted.

## VIII. Immune Reactions

The immune reactions of luminous bacteria are interesting from several points of view. First, it is known that *Cypridina* luciferase is antigenic, and the luminescent reaction is suppressed in the presence of homologous antiserum, though not in normal serum (102). Second, the readiness with which osmotic cytolysis occurs offers a means of testing the antigenicity of cell extracts, as well as the reactions of the antibodies to whole cells and to extracts with luminous cells, and with extracts and "ghosts" remaining after cytolysis. Third, the visible luminescence provides a means of noting changes in the metabolism of the organisms during such immune reactions as agglutination. Finally, the immune reactions offer a means of aiding in distinguishing and identifying species among a scattered group of morphologically related and unrelated bacteria whose nomenclature and synonyms are in a sad state of affairs.

The agglutination reactions of whole cells and different species or strains of luminous bacteria have been the subject of a few studies (13,150,155,163). The addition of either normal or immune serum increases the intensity of luminescence, most probably through the presence of metabolizable substrates. For the same reason, oxygen consumption increases, as shown by the decrease in time required for the organisms to use up the dissolved oxygen, thereby extinguishing the light. In strongly agglutinated cells, of course, the luminescence diminishes because of interference with the free diffusion of dissolved oxygen to cells within the clumped mass. According to Ninomiya (163), a further reduction in luminescence occurs in a mixture of complement plus antiserum, due to an actual injury of the bacteria.

The author has shown (115) that the immune serum to whole cells specifically agglutinates both whole cells and cytolysed ghosts, and specifically precipitates the Berkefeld-filtered cytolysates. An extensive study has been carried out by Warren (189) with eight species whose bacteriological characteristics were reinvestigated at the same time. Noteworthy results included the fact that cross agglutination occurred among bacteriologically diverse species. The Berkefeld filtrates of cytolysates proved to be antigenic and gave rise to agglutinins with specificities characteristic of the whole cell agglutinins. In cross precipitation between both filtrate antisera and whole cell antisera, and the filtrate antigens, it appeared that a mosaic of common antigens is present among the different species, and an intensified group reaction was found with the filtrate antisera. None of the antisera affected the luminescence of the living cells. From this it follows that either no antibodies were formed against the bacterial luciferase, or if they were present, they did not have access to the light-emitting system of the intact cells.

## IX. Addendum

The following papers which bear on the discussion in this review have become available to the author since the manuscript was submitted.

The reversible heat inactivation of *Cypridina* luciferase has been demonstrated in a quantitative study by Chase (44a) using highly purified luciferin and partially purified luciferase.

An extensive analysis of absorption, fluorescent, and chemiluminescent spectra has been carried out by van der Burg (183a) with dimethyldiacridylum nitrate, methylacridon, aminophthalic hydrazide and several derivatives, and pyromellitic acid hydrazide. Substituent groups in amino-

phthalic hydrazide influence both the intensity of fluorescence and the wave length of maximum luminescence, e.g., the maximum chemiluminescence of the unsubstituted compound occurs at 4125 Å, while that of the 3-nitro substituent occurs at 4020 Å, and that of the 3-amino substituent at 4240 Å. The emission spectrum of *Photobacterium phosphoreum* was reinvestigated, along with that of *P. splendidum*. Though the spectral distributions are closely related, the maximum intensity of the former species occurs 150 Å nearer the violet, and it is concluded that the emitting molecules are not identical in the two species. Moreover, the secondary maximum at 5400 Å reported by Eymers and van Schouwenburg (64) in the emission spectrum of *P. phosphoreum* was not observed, and the wave length of maximum intensity was found to be influenced by the thickness of the bacterial suspension, viz., a certain low range of cell densities indicated the same spectrum, while with heavier suspensions the maximum appeared to shift to slightly longer wave lengths, although the spectrum above 5500 Å remained the same. The photochemical inactivation spectrum (see 137,184 and the discussion on page 228) was also studied in the two species.

The emission spectrum of three luminous fungi, *Armillaria mellea*, *Mycena polygramma*, and *Omphalia flavida* were found to be so nearly identical that the light-emitting molecule must be considered as probably the same in each case. The maximum intensity lies in the green at 5250 Å. The spectrum extends to about 4700 Å in the direction of the violet, and to longer wave lengths than bacterial luminescence in the opposite direction.

In connection with the hypothesis that bacterial luciferin is a derivative of 1,4-naphthoquinone (134), Spruit and Schuiling (175b) have studied the action of various naphthoquinones on luminescence and rate of oxygen consumption of *P. phosphoreum*. In addition, Spruit (175a) has investigated the absorption spectra of a number of derivatives of naphthoquinone and related substances, with results favoring the view that bacterial luciferin consists of 1,4-naphthohydroquinone containing a side chain with ketohydroxy group substituted at position 2. In suspensions of luminous bacteria, addition of  $5 \times 10^{-6}$  M naphthohydroquinone caused a 50% inhibition of luminescence, under the experimental conditions used, while a corresponding inhibition of the total respiratory rate required  $1.6 \times 10^{-6}$  M. The effect was about inversely proportional to the number of bacteria, showing that the cells almost completely absorb the inhibitor from the medium. The action of several derivatives and redox indicators (methylene blue, pyocyanine, phthiocol, and others) was similar in inhibiting

luminescence much more than respiration. Urethan shares this characteristic, but differs with respect to the combined effect of cyanide. The naphthoquinone inhibition is counteracted by cyanide, whereas the urethan inhibition is increased. The data indicated that the naphthoquinones, etc. are dehydrogenated by a cyanide-sensitive system, and reduce luminescence by acting as hydrogen carriers, shifting the bacterial luciferin into a more oxidized state. A modification of the van Schouwenburg scheme (page 231) was therefore proposed, to include a pathway of luciferin dehydrogenation by the heme enzyme. The normal redox potential ( $E_0$ ,  $pH = 7$ ) of bacterial luciferin was estimated, on the basis of this study, as of the order of  $-50$  mv.

Interesting effects of calcium phosphate on bacterial luminescence have been reported in abstract by Schneyer (171a). Small concentrations of calcium and phosphate ions together, though not separately, increase luminescence throughout the temperature range of light emission in *P. phosphoreum*, an effect augmented under a pressure of 6000 lb. per sq. in. These ions, when simultaneously present, oppose the reversible and the irreversible thermal denaturation of the enzyme, and reverse the inhibition caused by quinine, which has been shown to be mediated through the reversible denaturation (132). Calcium phosphate appears to be an effective "renaturing agent" for the denatured form of the protein.

Giese (81a) has studied at some length the action of azide on luminescence, respiration, and growth of luminous bacteria. Growth is apparently much more sensitive than respiration or luminescence. Concentrations as high as  $0.1 M$  azide almost immediately and completely quench luminescence. (This is probably a true quenching; see page 225.) The same concentration reduces respiration only to about 10% of the control rate, suggesting a residual azide-insensitive respiration. Giese reports also that both luminescence and respiration are reduced in borate buffer, as compared with phosphate, at  $pH$  8.0 (81b). The significance of the buffer system and concentration has been referred to on page 241. Data with respect to luminescence in phosphate, phthalate, and acetate buffers have been discussed (126).

Of interest in connection with the discussion on pages 249 to 250 is a recent publication by Eyring, Johnson, and Gensler (67a) on the activity of extracted yeast invertase in relation to pressure, temperature, and  $pH$ . The reduction in activity on either the acid or alkaline side of the optimum  $pH$  of 4.5 is partially reversible by a hydrostatic pressure of 680 atmospheres. Analysis of the quantitative relation between increase in activity

with increase in pressure at  $pH$  7.02 to 7.07, and at  $35^{\circ}$  and  $40^{\circ}$  C., respectively, indicated that the reduced activity is brought about through a denaturation equilibrium characterized by a volume increase of 69 cc. per mole in going from the native, active to the denatured, inactive form. Unlike the luminescence system, and certain digestive enzymes (23), the reaction rate at room temperature and optimum  $pH$  was not decreased under pressure. A study of the action of urethan on invertase activity is in progress.

The relation of temperature and concentration of urethan to the rate of oxygen consumption and of methylene blue reduction by *Rhizobium trifolii* has been investigated by Koffler, Johnson, and Wilson (137a) with results in general similar to those obtained in studies of luminescence. The enzyme systems concerned in both methylene blue reduction and oxygen consumption, with glucose as substrate, undergo a reversible thermal inactivation, and the quantitative relation between the rates of either process and temperature, in both the control and urethan-containing suspensions, was calculated with some accuracy on the basis of the theory developed in connection with luminescence. Discrepancies between the theoretically calculated and the observed rates occurred, as in luminescence, at the high temperatures or strong concentrations of urethan which cause irreversible as well as reversible inhibition.

A more general and detailed treatment of the theory discussed in this review with reference to the relation between temperature and enzyme activity has been worked out by Morales (157a).

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# HEME-LINKED GROUPS AND MODE OF ACTION OF SOME HEMOPROTEINS

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## I. Introduction

In the course of the past ten years several of the enzymically active hemoproteins have been produced in a crystalline pure or an almost pure state (see Table I). Their production in pure state has made it possible also to begin to tackle the problem of the connection between chemical constitution of these hemoproteins and their activity. The investigations carried out previously on the easily procurable hemoglobin have in many

TABLE I

Hemoprotein	Source	Isolated by	Year	Ref. No.	Per cent of		Activity*	State
					Iron	Hemin		
Cytochrome c	Beef or horse heart muscle	Theorell	1935	69	0.34			Amorphous
		Keilin and Hartree	1937	41	0.34			Amorphous
	Horse-radish	Theorell and Åkeson	1939	82	0.43			Amorphous
		Elliott and Keilin	1934	27		1.05	818 (P.N.)	Amorphous
		Theorell	1941	73		1.48	1000 (P.N.)	Crystalline
Verdoperoxidase	Leucocytes	Agner	1941	5	0.1		75 (P.N.)	Amorphous
Cytochrome c	Yeast	Abrams, Altschul, and Hogness	1942	1		1.0	1700-2700 (Q)	Amorphous
Lactoperoxidase	Milk	Theorell and Åkeson	1943	85	0.07		72 (P.N.)	Crystalline
Catalase	Liver	Sumner and Dounce	1937	63			43,000 (K.f.)	Crystalline
		Sumner and Dounce	1937	62	0.06 to 0.1		26,000 (K.f.)	Crystalline
Catalase	Horse liver	Sumner and Dounce	1939	64		0.5	35,000 (K.f.)	Crystalline
		v. Euler and Josephson	1927	28	0.062 (calc.)	0.6	40,000 (K.f.)	Amorphous
Catalase		Agner	1938	3	0.085 ± 0.005		55,000-60,000 (K.f.)	Amorphous
		Agner	1939	4			62,000 (K.f.)	Amorphous
Catalase		Dounce and Frampton	1939	26	0.2	0.9	50,000-55,000 (K.f.)	Crystalline
		Sumner, Dounce, and Frampton	1940	65	0.092 to 0.094		22,000-40,000 (K.f.)	Crystalline
Catalase	Lamb liver	Agner	1942	6	0.093		60,000 (K.f.)	Crystalline
		Dounce	1942	25	0.1 to 0.2		30,000 (K.f.)	Crystalline (impure)
Catalase	Beef erythrocytes	Laskowski and Sumner	1941	48	0.12		48,000 (K.f.)	
Catalase	Horse erythrocytes	Agner	1941	7	0.087		65,000 (K.f.)	Amorphous
Catalase	Human erythrocytes	Bonnichsen	1947	13	0.093	1.08	65,000 (K.f.)	Crystalline
Catalase		Bonnichsen	1947	13	0.077	0.83	50,000 (K.f.)	Crystalline
Catalase	Human liver	Bonnichsen	1947	14	0.090	0.87	45,000 (K.f.)	Crystalline

\* P.N. = Peroxidase Number. Q = units per milligram. K.f. = "Katalasefähigkeit."

cases served here as a model, even though it has often proved necessary to modify the methods because of the scarcity of material.

Hemoglobins, cytochromes, peroxidases, and catalases have this feature in common—that their effect is centered in one or several iron atoms in each molecule. The iron atoms themselves mediate the reactions typical for each hemoprotein. As far as is known, the iron cannot be replaced by any other metal without loss of effect. Gjessing and Sumner (31) state that they once found a peroxidase effect in manganese protoporphyrin + peroxidase protein. This result, however, was not confirmed by Theorell (80). In all these proteins the iron is incorporated in the center of a porphyrin molecule whose four central nitrogen atoms are bound to the iron, thus blocking four of its six co-ordination places.



The heme thus formed has the shape of a flat disk with the iron in the center and its two remaining valencies directed at right angles from the plane of the disk. In the hemoproteins one or both are in all probability bound to the protein component.\* Under certain conditions, *e.g.*, in acid or alkaline solution, a reversible or irreversible splitting of one or both iron bindings to the protein component may take place. In this connection, however, heme is not always entirely freed from the protein component, but may remain bound by other groups than the iron.

\* In a private communication R. Lemberg has recently proposed certain changes in the nomenclature that will partly be used in this paper. For example, the Clark-Drabkin nomenclature for simple hematin compounds will be followed. Furthermore, the following generic terms will be used:

heme	= ferroprotoporphyrin
hematin	= ferriprotoporphyrin
hemochrome	= hemochromogen
hemichrome	= parahematin
hemoglobin	= hemoglobin
hemiglobin	= methemoglobin

In view of the Greek origin of the word cytochrome, it seems to us inadvisable to substitute "cytichrome" for ferricytochrome; "ferri-" and "ferro-" will therefore be used as prefixes to cytochrome. "Hemoproteins" will be used for all iron porphyrin proteins, irrespective of whether they contain ferrous or ferric iron.



Hemoproteins react in four different ways: (1) *Cytochromes* exercise their effect through the oscillation of the valency of the iron between ferrous and ferric,  $\text{Fe}^{3+} + e \rightleftharpoons \text{Fe}^{2+}$ . Since electrons are received from one quarter and given off to another, cytochromes thus transport a stream of electrons in a direction leading from hydrogen-transferring enzymes toward molecular oxygen. (2) In *hemoglobin* and *myoglobin* the iron remains, during physiological functioning, at the ferrous stage; oxygen is attached reversibly, without oxidation of the iron in this connection:  $\text{Fe}^{2+} + \text{O}_2 \rightleftharpoons \text{Fe}^{2+}\text{O}_2$ . (3) In *catalases*, according to the work of Sumner (61) and of Theorell and Agner (81), the iron remains trivalent the whole time, so that hydrogen peroxide would thus be bound to  $\text{Fe}^{3+}$  and there break down, probably by reaction with another molecule of hydrogen peroxide, to form  $\text{O}_2 + 2 \text{H}_2\text{O}$ . Keilin and Hartree (42) have advanced a theory opposed to this, according to which a change of valency in the iron enters in the catalase function (see page 299). (4) In *peroxidases* the iron remains trivalent during ordinary functioning (see, however, page 295). In a first reaction phase hydrogen peroxide is bound to the ferric atom, after which the substrate is oxidized by the peroxidase-hydrogen peroxide compound.

Why is it that the iron reacts so differently in different cases? The essential differences are not due to the chemical nature of the porphyrin. Hemoglobin, catalases, horse-radish peroxidase and cytochrome peroxidase all contain hematin as the prosthetic group; the verdo- and lactoperoxidases contain green iron porphyrins of unknown composition, but nevertheless react as peroxidases.

From this one can at once conclude that the essential differences must derive from the protein component. It is furthermore logical to assume that different parts of the very large (in relation to iron porphyrin) protein molecule must differ in importance in the determination of the mode of reaction of the iron. And, finally, it seems to be only reasonable to assume that the groups in the protein that are bound to the iron atom should have the greatest influence on it. A certain influence may further be expected from the coupling of protein groups to side chains in the porphyrin. It is also conceivable that groups situated near the iron atom without being coupled to it by chemical bindings may be of significance for its mode of reaction.

In accordance with this theory, one has to seek the cause of the different modes of reaction of the hemoproteins mainly in their heme-linked groups. A number of methods may be resorted to for the determination of the nature of these groups:

(1) Determinations of magnetic moment give information concerning the mode of binding of the iron atoms by giving the number of odd electrons therein. (2) Spectrophotometric determinations give information about dissociation constants for certain heme-linked groups. In the majority of cases the dissociation state of groups bound to or situated near the iron atom act upon the light absorption, but this is by no means always the case. Absorption bands of ferriporphyrin proteins show variation with *pH* more frequently than those of corresponding ferrous compounds. (3) Titrimetric determinations are used for comparison between the titration curves for, *e.g.* (a) the oxidized and the reduced form (cytochrome *c*, hemoglobin), (b) the reduced and the oxygenated form (of hemoglobin), or the reduced and the carbon monoxide compound (peroxidase), and (c) the protein component before and after coupling with hematin (peroxidase). In this way one obtains direct information concerning heme-linked groups, whose titration is changed by (a) change of valency in the iron, (b) change in mode of binding of the iron: ionic  $\rightleftharpoons$  covalent, and (c) coupling of hematin to the protein component. (4) Measurements of the redox potential at different *pH* values have so far been carried out only incompletely with hemoproteins, but have hitherto with hemoglobin and cytochrome *c* given data concerning dissociation constants for some heme-linked groups that are not spectrophotometrically demonstrable. The change of valency  $\text{Fe}^{2+} \rightleftharpoons \text{Fe}^{3+}$  affects the dissociation constant for a group situated sufficiently near the iron atom, whereby dependence on *pH* of the redox potential is changed according to known formulas. (5) Amino acid analysis of the protein component gives data concerning the amino acids that are present and that may thus conceivably be bound to the iron porphyrin. (6) Determinations of enzyme activity at different *pH*, or in the presence of inhibitors, have in a number of cases given information concerning which hemelinked groups are of importance for the enzyme function (*e.g.*, horse-radish peroxidase and catalases).

Generally speaking, it may be said that all the results so far obtained support the theory advanced, namely, that *the differentiation of the hemoproteins may be ascribed to their heme-linked groups*. This applies to the general mechanism of their effect, insofar as it has been possible to show essential differences between the mode of linkage of the heme in hemoglobin, cytochrome *c*, and peroxidases; concerning catalases, the only thing we know so far is that a hydroxyl is bound to the active iron atom, as is also the case with the peroxidases.

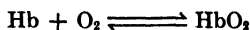
#### **Hemoproteins with One or Several Heme Groups per Molecule.**

Hemoglobins and catalases contain four atoms of iron per molecule, while cytochrome *c* and the peroxidases (horse-radish and the lactoperoxidases; the molecular weight of the others has not yet been determined) contain one iron atom per molecule. For hemoglobin, this means that the hemes within the same molecule exercise considerable interaction, which is of essential importance for the function of hemoglobin. Whether this is also the case in the catalases is not known. In hemoproteins with one iron atom per molecule (peroxidases and cytochrome *c*) it is evident that no intra-

molecular interaction can occur, which makes it easier to interpret experimental results.

## II. Hemoglobin

The physiological function of hemoglobin (Hb) is the reversible attachment of oxygen gas:



According to this equation, saturation of hemoglobin with oxygen at different pressures should give a hyperbolic curve, as is the case for myoglobin. The saturation curve of hemoglobin, however, is S shaped; this is due to interaction between the four heme-groups of the hemoglobin molecule, while the myoglobin has only one heme molecule. That the molecular weight of hemoglobin is four times the minimal value calculated from the iron content was shown by Adair (2) through osmotic measurements, by Svedberg and Fåhræus (67) and Svedberg and Nichols (68) through ultracentrifugation.

Adair advanced the theory that oxygen was attached successively to the four heme groups with different equilibrium constants, and the S-shaped curve could thus be represented by an equation with four constants, whose values changed in a regular manner with pH. Ferry and Green (29) arrived at similar results after careful experimental determinations of the form of the curve. Pauling (53) derived an equation with only two constants, on the assumption that all four heme groups were of equal value and interacted with one another as if they were placed at the corners of a square:



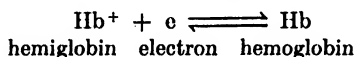
Each group would thus interact with two others, but not with the third. Pauling's equation is:

$$Y = \frac{K'p + (2\alpha - 1)K'^2p^2 + 3\alpha^2K'^3p^3 + \alpha^4K'^4p^4}{1 + 4K'p + (4\alpha + 2)K'^2p^2 + 4\alpha^2K'^3p^3 + \alpha^4K'^4p^4}$$

where  $Y$  is degree of saturation with oxygen,  $p$  is oxygen pressure,  $K'$  is related to the free energy change ( $= RT \ln K'$ ) accompanying the addition of oxygen to heme, and  $\alpha$  is related to the additional free energy ( $= RT \ln \alpha$ ) stabilizing two interacting  $\text{HbO}_2$  groups.

With a suitable selection of the values of  $\alpha$  and  $K'$ , complete agreement was obtained with the values of Ferry and Green. Equally good agreement was obtained, certainly, through the assumption that the heme groups were situated at the corners of a tetrahedron; but this possibility was rejected, as the heme groups, which must be assumed to be placed on the surface of the molecule, would then be too far distant from one another. The other conceivable alternatives, interaction between two and two in pairs, and no interaction at all, did not agree with the experimental data.

Coryell, Pauling, and Dodson (23), in connection with magnetic measurements during successive deoxygenation of oxyhemoglobin with hydrosulfite, found no evidence for interaction between the hemes (in which iron is bound with essentially ionic bonds, see below). Coryell (21) calculated the total effective interaction energies ( $4RT \ln \alpha$ ), arriving at the figures 3360, 3120, 0, and 0 cal. per mole for the equilibria of the hemoglobin with hydrosulfide, azide, fluoride, and hydroxide ions, respectively. In the two first-mentioned cases complexes with covalent bonds are formed; in the two last-mentioned the iron is bound with essentially ionic bonds. The absence of interaction in the two latter cases, however, is only apparent. By means of mathematical calculations on the basis of other authors' determinations of the oxidoreduction potential in the system:



Coryell was able to show "that interactions occur in this system of hemoglobin compounds, both of which contain ionically bound iron, and that the interaction energy effect of approximately 2600 cal. per mole is involved."

The physiologically important S shape of the saturation curve of hemoglobin is thus theoretically explained. It is, however, not yet known whether the heme groups are really placed at the corners of a square. The x-ray analyses of hemoglobin crystals by Boyes-Watson and Perutz (15) seem to indicate that the hemoglobin molecule is built up of four equal and parallel layers which form a platelet having the dimensions  $36 \times 64 \times 48 \text{ \AA}$ . "X-ray data showed that the four heme groups in methemoglobin must be arranged in pairs related by twofold axis. Their parallelism now indicates that they may lie at the corners of a square as suggested by Pauling." (See Perutz, 54a).

When oxygen is attached to hemoglobin in neutral solution, the  $pH$  of the solution is lowered (the Bohr effect). This phenomenon is of great physiological importance for the transport of carbon dioxide in the blood.

It is only through the explanation of the chemical nature of heme-linked groups in the hemoglobin that it has become possible to understand this effect.

Many different methods of investigation have been resorted to in order to solve this problem. Of great importance was the analogy between oxy-hemoglobin and the hemochromes; the latter have two nitrogen-containing groups, *e.g.*, pyridine, nicotine, picoline, or histidine coupled to the iron atom in ferroporphyrin. The hemochromes show absorption spectra of a type resembling oxyhemoglobin with two strong absorption bands in green. The constitution of the hemochromes was explained through the studies of Anson and Mirsky, Conant *et al.*, Barron and Hastings, and in a great work by Clark, Taylor, Davies, and Vestling (17). According to Clark *et al.*, two molecules of water are bound to the iron in heme; in hematin, on the other hand, one molecule of water and one hydroxyl group are bound to iron:



In heme both water molecules can easily be replaced by a nitrogen base or by cyanide ions. In hematin the water molecule is replaced by nitrogen bases, though less easily than in heme, while the hydroxyl group is not replaced by the nitrogen base, though it is by cyanide ions, as is the case with the water molecule. The relation between the dissociation constants for the binding of the nitrogen bases and the cyanide ions to heme and hematin, respectively, determines the value of the oxidation-reduction potential. Since the binding of the nitrogen bases to heme is stronger than that to hematin, the oxidation-reduction potential is raised when nitrogen bases are coupled to heme and hematin.

Determinations of the dependence of the oxidation-reduction potential upon the *pH* of the solution are in many cases able to give data concerning the dissociation constants for heme-linked groups according to the general equation:

$$E'_0 = E_0 + (RT/F)[\Sigma \ln (K_r + \text{H}^+) - \Sigma \ln (K_o + \text{H}^+)]$$

where  $K_r$  = the dissociation constant for the reduced or ferrous form and  $K_o$  = the dissociation constant for the oxidized or ferric form.

The majority of dissociable groups of the protein part have the same

values for  $K$ , and  $K_0$ , and thus do not affect  $E'_0$ . Only groups in the vicinity of the iron atom, thus heme-linked groups, give differences as a result of changes in the oxidation state and therewith in the charge of the iron atom. The possibility of determining  $pK$  for all heme-linked groups by this method is restricted by the fact that, if the dissociation constant of one heme-linked group in the ferrous form happens to lie in the vicinity of another one in the ferric form, these two cancel each other's effect, so that neither becomes apparent. In practice, a difference of about 0.2 unit in  $pK$  is required between groups in the ferro and ferri forms to give a clear effect on the oxidation-reduction potential.

The first determinations of the hemo-hemoglobin electrode potential were carried out by Conant and his co-workers (18). Conant and Pappenheimer (19) further determined the potential spectrophotometrically in oxidation equilibria. At  $pH$  6.9 and  $26^\circ$  they found the value of  $E_0 = +0.152$  v. Havemann and Wolff (35) observed a break in the curve  $E_0$  plotted against  $pH$ , the  $E_0$  being practically independent of  $pH$  on the acid side of  $pH$  6.5, though it sloped down at higher  $pH$  values. Hastings and Taylor (33), in a thorough investigation of the oxidation-reduction potentials at different  $pH$  values, were able to establish the existence of a heme-linked group in hemoglobin with a dissociation constant  $K_1$ , of  $2.24 \times 10^{-7}$  ( $pK_1 = 6.65$ ) at  $30^\circ C$ . The potential of the hemo-hemoglobin system in the  $pH$  region 5 to 9 and  $30^\circ$  was given by:

$$E'_m = E_m + 0.0601 \log \frac{[H^+]}{K + [H^+]}$$

where  $E_m = 0.168$  v., and  $K = 2.24 \times 10^{-7}$ . In his Harvey Lecture in 1932 Conant stated:

"There are some indications that the histidine grouping may be involved in the linkage of the globin to the iron, but this aspect of the hemoglobin problem is still completely unsolved."

#### A. TITRIMETRIC INVESTIGATIONS

Wyman and collaborators presented conclusive experimental evidence that Conant's assumption was justified—that imidazole residues of histidine must be linked to the iron in hemoglobin. The differential acid-base titration data of German and Wyman (30) between hemoglobin and oxyhemoglobin were taken as the base for calculations by Wyman (92), and these established the existence in hemoglobin of two acid groups ( $pK$  5.25 and 7.81). On oxygenation, these values shifted to 5.75 and 6.80.

Since the apparent heat of dissociation of the groups titrated between  $pH$  5.5 and 8.5 fitted the values ( $Q = 6500$  cal. per mole) for the imidazole nucleus, Wyman drew the conclusion that in hemoglobin, histidine residues are linked to the iron. Wyman and Ingalls (93) extended the differential titrations to hemi- and hemoglobin, and found the following  $pK$  values at  $25^\circ C.$  and at ionic strength  $\mu = 0.16$ :

Hb	HbO <sub>2</sub>	Hb <sup>+</sup>
5.25.....	5.75.....	5.75
7.93.....	6.68.....	6.68
— .....	— .....	8.01

Theorell's (79) differential titration of horse hemi- and hemoglobin at  $20^\circ$  and low ionic strength yielded the values:

Hb	Hb <sup>+</sup>
5.2.....	5.45
7.8.....	6.5
— .....	8.1

These values are in satisfactory, though not complete, agreement with the Wyman-Ingalls values, which, owing to war conditions, were unknown to the author.

The titration differences between globin and hemoglobin in the  $pH$  region 5.5 to 11.3 at  $0^\circ$  indicated the presence in globin of a group with  $pK$  10 that was not present in the  $pH$  range accessible to titration in hemoglobin. As a possible explanation, it was suggested that a positively charged group, *e.g.*, a primary amine group, was in combination with heme linked to one of its propionyl groups. A similar effect seems to occur in the horse-radish peroxidase (see page 293).

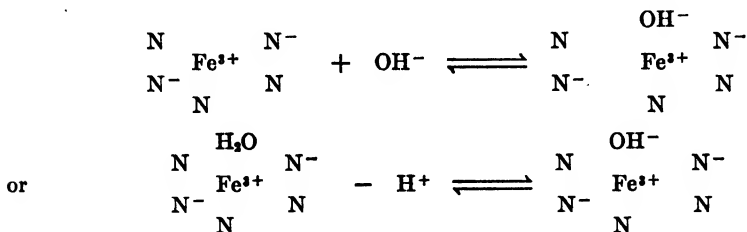
The titration difference between globin and hemoglobin was found to be one equivalent less than calculated. Different explanations are possible, but are still without experimental support.

## B. SPECTROPHOTOMETRIC INVESTIGATIONS

The state of ionization of the imidazole residue linked to hemoglobin iron has no hitherto observed influence on the light absorption of hemi-, hemo-, or oxyhemoglobin. It seems possible, however, that very careful spectrophotometric determinations might reveal slight differences corresponding to the  $pK$  values of the heme-linked imidazole groups.

Hemoglobin in neutral solution is brownish in color, in alkaline solutions red. This change in color has been subjected to spectrophotometric measurements by Haurowitz (34) and by Austin and Drabkin (10). The

latter authors found that the transition follows a simple dissociation curve with  $pK = 8.12 \pm 0.01$  at the ionic strength 0.10, and that it arises from the addition of a hydroxyl group to the iron, or perhaps rather the removal of a hydrogen ion from a water molecule linked to the iron:



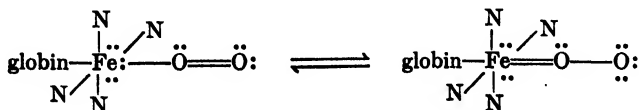
### C. MAGNETOMETRIC MEASUREMENTS

Coryell, Stitt, and Pauling (24) determined the magnetic moment of hemoglobin at different pH values, and of some of its derivatives. The following values were obtained: hemoglobin, 5.80 Bohr magnetons at pH 6, but decreased slightly at pH 5; hemoglobin hydroxide, 4.47; hemoglobin fluoride, 5.92; hemoglobin cyanide, 2.50; and hemoglobin hydrosulfide, 2.26.

In hemoglobin and its fluoride compound the type of linkage is thus essentially ionic, with five unpaired electrons per iron atom. The value for the hydroxide corresponded to three unpaired electrons, indicating bonds of an intermediate type, whereas the bonds in the cyanide and the hydrosulfide are essentially covalent.

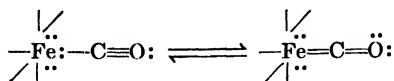
The value of  $K = ([\text{Hb}^+][\text{OH}^-])/[\text{Hb}[\text{OH}]]$  found by the magnetic measurements agreed within the limits of error with the results of Austin and Drabkin results. Furthermore, fluoride ion was found to compete with hydroxyl ion for the same linkage to hemoglobin.

In 1936 Pauling and Coryell (54) made the important discovery that the iron in hemoglobin is held by essentially ionic bonds. On oxygenation or poisoning with carbon monoxide the type of bond changes to essentially covalent. This involves a profound change in the electronic structure of the oxygen molecule on combination with hemoglobin, as the free oxygen molecule contains two unpaired electrons. The authors consider the following resonating structures of oxyhemoglobin to be the most probable:

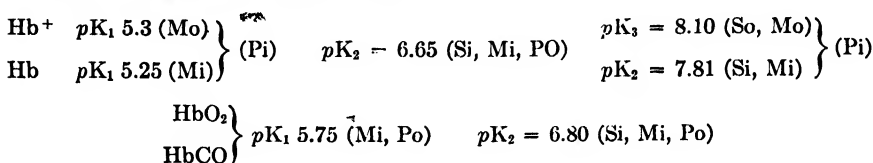




The electronic structure of carboxyhemoglobin is supposed to be analogous:

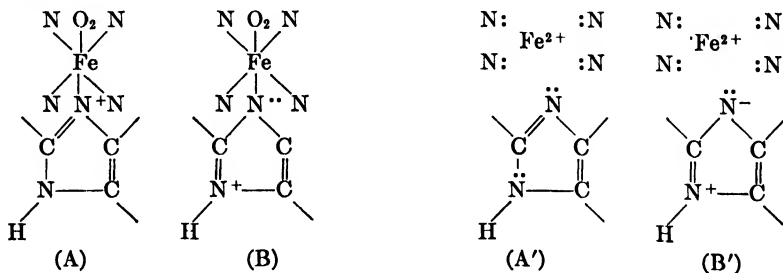


Coryell and Pauling (22) have attempted a structural interpretation of the heme-linked groups in hemoglobin and its derivatives on the basis of the evidence mentioned above. They summarize the heme-linked groups in the pH range 4.5 to 9 as follows:



where M = magnetometrically, P = potentiometrically, S = spectrophotometrically, i = inoperable, and o = operable.

The  $pK_3$  value of  $\text{Hb}^+$  (= 8.10) corresponds to the addition of a hydroxyl group; the "third acid group" is thus the ferric atom itself. The value 7.81 for the  $pK_2$  of Hb corresponds to the titration of an imidazole nucleus comparatively firmly linked to the iron by essentially ionic bonds. The difference between the  $pK_3$  of 8.10 for  $\text{Hb}^+$  and the  $pK_2$  of 7.81 for Hb is small enough to bring about a practical cancellation of their effect on the oxidation-reduction potential. On oxygenation of Hb to  $\text{HbO}_2$ , the  $pK_2$  changes from 7.81 to 6.80. This is explained by Coryell and Pauling in the following way:



The resonating structures A and B are considered to contribute nearly equally to the normal state of the molecule, and the resulting  $pK$  must therefore occur somewhere in the middle, between the  $pK$  of a pyridinium

group (like B) with a  $pK$  in the neighborhood of 5, and a dimethylamine-like structure (A) with a  $pK$  far in the alkaline range. The observed  $pK$  value, 6.80, agrees reasonably well with these facts. In Hb the structures A' and B' are not equivalent, since B', with separated charges, is less stable than A', and thus gives a smaller contribution than A' to the normal state of the molecule. The  $pK$  of the imidazolium group must for this reason be higher in the ionic than in the covalent structure, in accordance with the experimental evidence; "hence it is predicted with certainty that the change in bond type for the iron atom accompanying removal of the oxygen molecule must be accompanied by a decrease in the acidity of the attached imidazole group."

The oxidation of hemo- to hemoglobin is accompanied by decrease in  $pK_2$  by 1.16 pH units, from 7.81 to 6.65. The distance between the iron atom and the titrable imidazole nitrogen group was calculated from these data to be about 5 Å, which agrees with the calculated distances and angles from iron to the second nitrogen of the ring.

The interpretation of the  $pK_1$  is somewhat more uncertain. A second imidazole group is supposed to be attached to the iron from the other side of the heme disc by a loose bond in Hb and in Hb<sup>+</sup>. The bond can be broken in Hb by the entrance of oxygen or carbon monoxide, or in Hb as well as Hb<sup>+</sup> by a hydrogen ion at high enough acidity. This affords an explanation of the low value, 5.25, of  $pK_1$  in hemoglobin. In oxyhemoglobin the oxygen molecule prevents the interaction of the displaced imidazole nucleus with the iron atom, so that the  $pK_1$  rises to a value in the neighborhood of the histidine itself. In hemoglobin the increase in formal charge by one might be expected to decrease the  $pK$  value. As this is not the case, Coryell and Pauling suggest that a water molecule co-ordinates through dipolar attraction more firmly to the ferric than to the ferrous iron. The expected decrease in  $pK$  following the oxidation would thus be completely cancelled by the entrance of a water molecule. This part of the theory may need further experimental evidence. It should be noticed that the dissociation constant of the nitrogen atom in position 3 of this loosely bound imidazole is not discussed at all in the paper of Coryell and Pauling.

The work referred to above affords reasonable possibilities of explaining two of the main features of the mode of action of hemoglobin: the sigmoid shape of the oxygen equilibrium curve, and the change in acidity on oxygenation of ferrohemo-globin. A third, most important feature, however, remains unexplained: we still cannot understand why hemo-

globin can take up oxygen reversibly without effecting oxidation of the ferrous iron to ferric.

Holden (36) has recently questioned the existence of linkages between the protein component and the iron in hemoproteins, and has also advanced the theory that carbon monoxide would be attached to the iron in hemoglobin, but that oxygen would be attached to some other part of the molecule. Holden's arguments cannot be reviewed and examined in detail here. It may, however, be pointed out that it seems impossible to explain, on the basis of Holden's theory, how the combination of hemoglobin with carbon monoxide or with oxygen could give the same Bohr effect. Generally speaking, many of the experimental data referred to above remain unexplained by Holden's theory, which seems to be based largely upon spectroscopic analogies.

### III. Cytochrome c

The material hitherto used for the production in the pure state of cytochrome c has been the cardiac muscle of the horse or cow. In 1935-36 Theorell obtained a preparation with 0.34% iron (69,70). In 1937 Keilin and Hartree (41) described an improved method of production, which gave a cytochrome preparation having the same iron content as that of Theorell. In 1939 Theorell and Åkeson (82,83) reported that these preparations were not homogeneous and could be purified by electrophoresis to an iron content of 0.43%. This was confirmed in 1945 by Keilin and Hartree (45), who obtained the same iron content through treatment with ammonium sulfate at pH 10. Keilin and Hartree are of the opinion that even the preparation with 0.34% iron may be regarded as pure, basing this view on the fact that preparations with the same iron content have been obtained both with Theorell's method and with that of Keilin and Hartree. The further purification to 0.43% iron would in their opinion imply that an inert part of the cytochrome molecule were split off. Against this it may be said, however, that no criteria of purity have been presented for the cytochrome with 0.34% iron. On the contrary, our electrophoretic experiments showed that it was not homogeneous on electrophoresis even below pH 10, although from the point of view of method it proved advantageous to undertake the electrophoretic purification at pH 10.65. "The preparation purified in this way" (with 0.43% iron), "unlike the preparation with 0.34% iron, migrates uniformly on electrophoresis at different hydrogen ion concentrations" (Theorell and Åkeson 1941).

Keilin and Hartree assert further that cytochrome with 0.34% iron is

probably pure, since it fits into Svedberg's multiple system of molecular weights, while 0.43% iron corresponds to a molecular weight of 13,000, "which is unprecedented among proteins." This argument we can by no means accept. Svedberg's multiple law gives expression to a general tendency, but exceptions to the rule are so numerous that the validity of the law, even in an approximative sense, has in a number of quarters been contested.

Thus "0.34% cytochrome" is definitely not homogeneous on electrophoresis, and we have no proofs of its homogeneity; the "0.43% cytochrome," however, is homogeneous on electrophoresis, and the amino acid analyses have so far not shown any definite deviation from integers in the ratio of moles amino acid to atoms of iron. Further criteria of purity would clearly be required before one could definitely assert that even this cytochrome c is chemically pure. Cytochrome c with 0.34% iron, on the other hand, is in our opinion definitely impure.

Keilin and Hartree tested the catalytic effect of the two cytochrome preparations with (1) cytochrome oxidase, cytochrome c, and ascorbic acid, and (2) cytochrome oxidase, cytochrome c, succinic dehydrogenase, and succinic acid without finding any difference per iron atom in the effect of the two cytochrome preparations. This, in our view, indicates that certain inert impurities (in these two systems) are removed on the final purification, rather than that 20% of the cytochrome molecule is split off in the purification.

And finally, it may be pointed out that the impurities in the "0.34% cytochrome" were, certainly, inactive in the two systems tested by Keilin and Hartree, where the factor deciding the rate of the reaction was of course cytochrome c. This naturally does not show that the impurities are

TABLE II  
AMINO ACIDS IN CYTOCHROME C (83)

Amino acid	Per cent found		Calculation for the number of amino acid given in last column	Probable number of amino acids per molecule
	Hydrolysis 1	Hydrolysis 2		
Histidine	3.22	3.27	3.57	3
Arginine	2.62	2.81	2.65	2
Lysine	22.1	24.7	24.5	22
Tyrosine	5.43		5.50	4
Tryptophan	1.21		1.55	1

without significance in all other systems; these must, on the other hand, be tested in each separate case.

The amino acid analyses in the purest preparations with 0.43% iron gave the results shown in Table II. Cystine, lysine, tyrosine, glutamic and aspartic acids, and leucine were isolated in a pure state from cytochrome c.

The sulfur content is 1.43%, corresponding to 6.0 atoms of sulfur per molecule. Two atoms of sulfur occur in methionine (9). Of the other four, two are contained in cysteine bound by thio ether linkages to the side chains (in the 2 and 4 positions) of the heme. The rather uncertain determination of cystine plus cysteine in cytochrome c gave between two and three sulfur atoms per molecule. One or two atoms of sulfur are thus still not accounted for.

#### A. SPECTROPHOTOMETRIC INVESTIGATIONS

Ferricytochrome c shows five slightly different absorption spectra, according to the pH of the solution (84). They merge reversibly into one another when the degree of acidity is changed, and it was possible spectrophotometrically to determine  $pK'$  for the merging of the different types into each other. See Figure 1. Only the dissociation curve between forms III and IV had the normal slope ( $n = 1$ ), whereas the others were more or less too steep ( $n > 1$ ).

Type I gives the same absorption spectrum as free "hematin c" in acid solution, with a strong band at 635  $m\mu$  and less pronounced bands at 542 and 505  $m\mu$ . The position of the Soret band was not determined for type I. Type II shows a spectrum intermediate between I and III; the band in red is less intense than in I, and is displaced to 622  $m\mu$ . Other absorption maxima are found at 525, 497, 395 (Soret band), and 275  $m\mu$ . Type III shows the previously well-known bands of ferricytochrome c in about neutral solutions: 695 and 655  $m\mu$  (visible only in strong solutions), 565, 530, 408, 365, and 280  $m\mu$ . Type IV gives bright red solutions because it has no bands in red and the general light absorption in red is lower than in any of the other types. Spectroscopic absorption maxima were found at 565, 537, 408, 355, and 280  $m\mu$ . Type V shows bands at 565, 536, 412, 350, and 290  $m\mu$ .

Type II gives a fluoride compound with a strong band at 601  $m\mu$ . It can exist only in a very narrow pH range around pH 3. The reason is that at lower pH values the hydrofluoric acid becomes more and more undissociated, whereas at higher pH values type II merges into III, which gives

no fluoride compound. Type V, but not the others, gave a cyanide compound. The two-banded spectrum was replaced by a single band in the green part of the spectrum, resembling the hemoglobin cyanide.

Potter (55) found cyanide inhibition of the reduction of ferri-cytochrome *c* by succinic dehydrogenase and dihydrocozymase. This inhibition, however, appeared only after incubation with cyanide, which seems remarkable, since a ferri-cytochrome cyanide would be expected to be formed rapidly. Recently Horecker and Kornberg (37) investigated the formation of ferri-cytochrome cyanide at pH 7.4 and 8.0, and confirmed Potter's observation. However, it takes an amazingly long time, seven hours at 27°C., for the equilibrium to be established, so ferri-cytochrome differs in this respect from all other known cytochrome compounds. It may be mentioned that the band at 692 m $\mu$ , described as new by the authors, were described in 1934 by Bigwood, Thomas, and Wolfers (12).

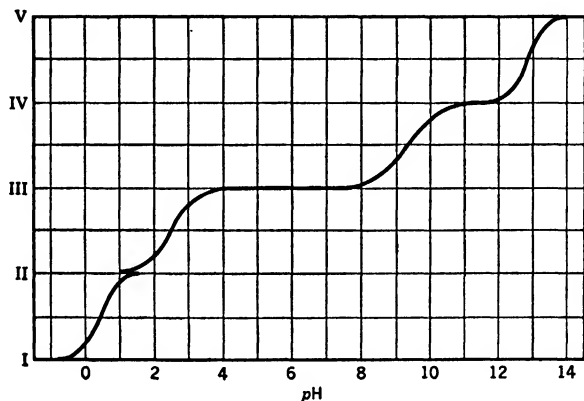


Fig. 1. Spectrophotometric titration curve of ferri-cytochrome *c* (84).  $pK$  values are: I-II, 0.42; II-III, 2.50; III-IV, 9.35; IV-V, 12.76.

*Ferrocyclochrome c* shows the same bands in the spectroscop over the entire  $pH$  scale. Precise spectrophotometric measurements of the light absorption at different acidities seem not to have been carried out. Unpublished experiments by K. G. Paul show that the absorption coefficient at 550 m $\mu$ , on top of the main visible band, changes its value somewhat between  $pH$  9 and 10, indicating a heme-linked group with  $pK$  in this region.

There are, however, also other reasons in support of the assumption that heme-linked groups in ferrocyclochrome are affected by changes in  $pH$ . Below  $pH$  4 and above  $pH$  13 ferrocyclochrome *c* is autoxidizable. Between  $pH$  3 and  $pH$  12 no carbon monoxide compounds are formed; but at  $pH$  2

the bands at 550 and 520  $m\mu$  are slowly replaced by a mixed spectrum of ferrocytochrome and its carbon monoxide compound (bands at 563 and 530  $m\mu$ ). At pH 1, on reduction in a carbon monoxide atmosphere, the bands at 550 and 520  $m\mu$  appear first, and are entirely replaced by the bands at 563 and 530  $m\mu$  in the course of about one minute. The addition of carbon monoxide is thus slow in acid solution. At pH 13 one gets a mixed spectrum of (550, 520) and (563, 530) immediately, whereas at pH 14, bands at 563 and 530  $m\mu$  appear immediately. The ratio of iron to carbon monoxide is one in alkaline solution; it has not been measured in acid solution.

The results of the differential titration of ferrous and ferric cytochrome c are shown in Figure 2. The difference remains constant at one equivalent

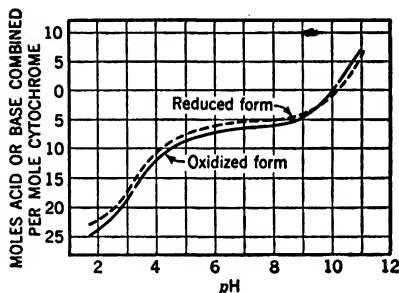


Fig. 2. Acid- and base-combining capacity of ferrous and ferricytochrome c at 20°C. (84).

per mole between pH 3.5 and pH 8. This difference arises from the formation of a hydrogen ion on the reduction with platinum-hydrogen:  $\text{Fe}^{3+} + \text{H} \rightarrow \text{Fe}^{2+} + \text{H}^+$ . Below 3.5 it increases up to two equivalents at pH 1.5. The heme-linked group with  $pK$  of 2.5 determined spectrophotometrically is thus titrated in ferricytochrome; but no corresponding group was found, either titrimetrically or spectrophotometrically, in ferrocytochrome. Above pH 8 the curves approach each other and intersect at about pH 9.6, so that the difference above pH 10 amounts to nearly one equivalent again. This would mean that two heme-linked groups in ferricytochrome were titrated. The corresponding groups in ferrocytochrome must be assumed to have their  $pK$  above the accessible pH range ( $> 11$ ). The result is puzzling, since only one of the groups in this region was found spectrophotometrically in ferricytochrome.

From pH 5.5 to pH 8.5, the normal region of titration of the histidine imidazole, a total of about two equivalents was titrated in both ferric and ferrous cytochrome. Not even the whole of these two equivalents, however, can be attributed to imidazole according to the determination of the apparent heat of dissociation with the aid of titrations at 0° and 20°C. On the strength of these data it was suspected that two of the three imidazole nuclei present in the cytochrome c molecule were linked to the iron in a way that displaced their  $pK$  values out of the normal range.

## B. MAGNETOMETRIC MEASUREMENTS

The magnetometric measurements showed that ferrocytochrome c and its carbon monoxide compound were diamagnetic over the whole pH scale, indicating that the iron is held by essentially covalent bonds. The ferricytochrome III gave values corresponding to one unpaired electron, the iron thus being held by essentially covalent bonds. Type II (pH 1.4) gave a molar susceptibility,  $\chi_{mol}$  of  $11,300 \times 10^{-6}$ , not far from the value of Coryell and Pauling (22) for hemoglobin in acid solution,  $\chi_{mol} = 12,570 \times 10^{-6}$ . The paramagnetic susceptibility of type I is still somewhat higher; the value  $\chi_{mol} = 13,060 \times 10^{-6}$  was obtained at pH 0.70. In extremely acid solution the value would probably approach the theoretical value for five odd electrons,  $\chi_{mol} = 14,800 \times 10^{-6}$  or 5.92 Bohr magnetons. In types I and II the iron is thus held by essentially ionic bonds.

The dissociation constant of ferricytochrome fluoride at pH 3.14 was determined magnetometrically. The value found,  $K = 0.0159$ , closely agrees with that of hemoglobin fluoride.

The ferricytochrome cyanide contained one unpaired electron (covalent bonds). It contains one cyanide per heme.

## C. OXIDATION-REDUCTION POTENTIAL

For the oxidation-reduction potential of the cytochrome c the following values have been reported:

Reference	pH	$E'_0$
Coolidge (20)	7	+0.260
Stone and Coulter (59)	7	0.280
Stotz <i>et al.</i> (60)	5-8	0.262
Wurmser and Filitti-Wurmser (91)	5-8	0.253
Ball (11) (muscles <i>in situ</i> )		0.27
Green (32)	7	0.124
de Toeuf (89)	7	0.120-0.180

The values of de Toeuf and of Green differ considerably from those of the others, and are probably erroneous, whereas the values from the other five publications agree reasonably well. The values, however, do not extend beyond the pH range of 5-8, and do not show any significant indication of a slope in this region.

In order to study the dissociation constants of heme-linked groups, it was obviously of great interest to determine the redox potential throughout the whole pH scale. In the author's institute, Paul has carried out experiments along these lines (52). Owing to experimental difficulties, the pH



region from 1 to 4 still remains unexplored. In the pH region from 4 to 7 the slope is either nil or very low, the values being slightly above  $+0.260$  v., which are in accordance with the investigations of earlier authors. At pH 6.86 a break occurs, so that the curve from pH 7.5 to 8.7 shows a  $dE_0'/dpH$  of about  $-0.060$  v.

This result indicates the existence of a hitherto unknown heme-linked group in ferricytochrome, with  $pK'$  0.86 (see Fig. 3).

#### D. DISCUSSION

In 1940 (72) the theory was advanced that both of the hemochrome-forming groups in cytochrome c were imidazoles belonging to two of the

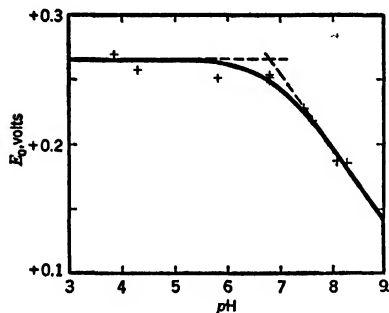


Fig. 3. Redox potential of cytochrome c, according to Paul (52).

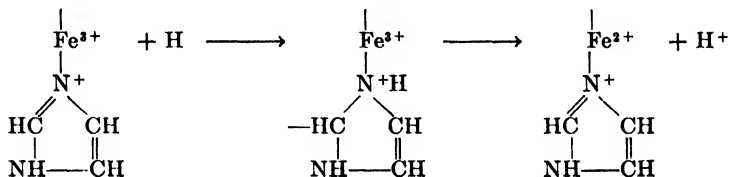
three histidine residues of the molecule. The main reasons for this assumption were the following: (1) The dissociation constants of the imidazoles, according to the titrimetric data, were partly displaced from their normal range. (2) The spectroscopic data showed several striking analogies between ferricytochrome of type II, in which one of the hemochrome-forming groups is dissociated off, and hemoglobin in acid solution, which has one imidazole group linked to the iron. Ferricytochrome cy-

nide and fluoride and carbon monoxide ferrocytochrome were analogous to the corresponding hemoglobin derivatives. (3) The paramagnetic susceptibility of type II was nearly equal to that of acid ferrihemoglobin. The dissociation constant of type II fluoride was equal to that of acid hemoglobin. (4) Titrimetric experiments indicated the presence of two heme-linked groups in ferricytochrome with their  $pK$  values between pH 9 and 10; the spectrophotometric determinations revealed only one of them,  $pK' = 9.35$ . Russell and Pauling (56) had carried out some magnetic determinations, at three different pH values, of the dissociation constant of the covalent imidazole hemoglobin complex. A new heme-linked group with  $pK = 9.5$ , which did not pre-exist in hemoglobin, was interpreted as the imino group of the imidazole. This interpretation seemed to offer a possible explanation for the heme-linked groups with  $pK$  values between 9 and 10 in ferricytochrome, namely, that these groups were histidine imidazoles.

This assumption explained also another peculiar fact, *i.e.*, how heme-linked groups could be titrated without breaking their bonds to the iron.

Certain features in the mode of action of the cytochromes were now readily understood. The heme disc is firmly fixed from both sides by the hemochrome-forming groups of the protein; the heme may be built into a crevice of the protein. No valencies of the iron, at physiological *pH*, are free to give compounds with carbon monoxide, cyanide, fluoride, etc. Ferrocyclochrome is not oxidized by oxygen, because the iron is not accessible to oxygen unless at least one of the protein groups is dissociated from the iron, below *pH* 4 or above *pH* 13.

On the other hand, cytochrome iron might be supposed easily to take up or give off an electron with the aid of the covalent bond to the imidazole nucleus, in which free radicals may appear:

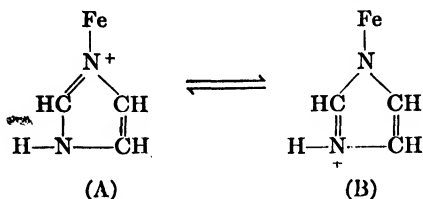


"In consequence of the ability of the imidazole nucleus to form structures resonating with one another in different ways we may conceive the radical formation to take place with the help of any of the four N atoms in the two imidazole rings, after which the exchange of electrons with the iron atom is easily effected thanks to the covalent linkages. Thus, although the iron atom is built into the cytochrome molecule in such a way that it is not accessible to the direct influence of oxidizing or reducing reagents, it becomes in this way open for oxidoreduction. The primary process in the imidazole nucleus is here completely analogous with Michaelis' 'one-step-reaction' in the oxidoreduction of riboflavin or the pyridine nucleotides. There would thus appear to be an unexpected similarity in the mode of reaction of the 'hydrogen-transferring' ferments and that of a hemin-proteid" (79).

Much additional information is still needed before the theory concerning the linkage of the iron in cytochrome to imidazole nuclei can be confirmed. It must not be forgotten that we still know very little about the dissociation constants of the heme-linked groups in ferrocyclochrome. The first few data, which are still incomplete, were determined recently by Paul. There are still several experimental possibilities left to complete the picture, and these are now being followed up. In the meantime, a detailed discussion is pointless. Only a few aspects may be mentioned. The new heme-linked group in ferricytochrome, with *pK* of 6.86 (Paul), fits in very well with the imidazole linkage theory, because a histidine imidazole group

linked to ferroheme by covalent bonds is expected to have its  $pK$  near 7. In oxyhemoglobin the corresponding  $pK$  is 6.80.

It may further be pointed out that there is a contradiction between Russell and Pauling (1939) (56) and Coryell and Pauling (1940) (22). The former attributed the heme-linked group,  $pK'$  9.5, in imidazole hemoglobin to the titration of the imino group (see formula A). Coryell and Pauling,



however, pointed out that considering the resonance of A with B one had to predict a  $pK$  of about 7 (see page 277) in oxyhemoglobin, which can be represented with the same formulas A and B, disregarding the difference that we are here dealing with a histidine imidazole instead of free imidazole. Theorell (72) pointed out that the interpretation of Russell and Pauling necessarily implied the assumption that for some unknown reason A made a larger contribution to the normal state of the molecule than B. But nobody has so far been able to explain why it should be so.

#### IV. Peroxidases

As can be seen from the Table I (page 266), four peroxidases have been produced in a pure or nearly pure state. Two of them have been crystallized, horse-radish peroxidase and lactoperoxidase; these are the only ones that have been submitted to any investigations concerning their heme-linked groups. Most of the work has been carried out with horse-radish peroxidase, which is comparatively easily available and has the great advantage that it can be split reversibly into prosthetic group and protein component (71).

##### A. COMPOSITION OF HORSE-RADISH PEROXIDASE

Horse-radish peroxidase consists of one molecule of ferriprotoporphyrin linked to a colorless protein. The molecular weight is 44,100 (74). Lactoperoxidase contains one molecule per molecule of a greenish ferriprotoporphyrin of unknown composition; the molecular weight is 93,000 (85).

A preparation of crystallized horse-radish peroxidase showed the following composition (78): C = 47.0%, H = 7.35%, N = 13.2%, S =

0.43%, Fe = 0.127%, and O (by difference) = 32.0%. Thus every molecule contains 416 atoms of nitrogen, 6.0 sulfur atoms, and 1.00 iron atom. On acid hydrolysis an insoluble humin residue of 18.4% appeared, probably deriving from acid carbohydrates in the molecule.

After submitting the hydrolyzate to microelectrodialysis the following analytical values were obtained: humin nitrogen, 1.1%; amide nitrogen, 13.0%; cathode nitrogen, 24.2%; neutral nitrogen, 45.1%; and anode nitrogen, 16.2%. The following values for amino acids were obtained: histidine, 0.71% of the weight (calculated for three molecules per molecule, 0.71%); arginine, 6.91% of the weight (calculated for eighteen molecules per molecule, 7.11%); and lysine, 4.06% of the weight (calculated for twelve molecules per molecule, 3.98%). The total calculated number of amino acids per molecule was 287, which, within the limits of error, agrees with the Bergmann-Niemann number,  $288 = 2^5 \times 3^2$ .

The *reversible splitting* of the horse-radish peroxidase can be performed by adding a cold water solution of the enzyme to an acetone-hydrochloric acid mixture (1 ml. concentrated hydrochloric acid per liter acetone) at  $-5^\circ$  to  $-10^\circ\text{C}$ . The protein precipitate in the course of some minutes becomes more and more decolorized; protohemin goes into solution. After centrifuging in the cold the colorless protein is dissolved in cold 1% sodium bicarbonate. If a solution of protohemin in a minimum of alkali is added to the protein the brown-greenish color of the hematin in half an hour turns the pure brown color of the peroxidase, and the activity is restored (71).

Theorell, Bergström, and Åkeson (86) investigated which of a number of other hemins were able to reactivate the peroxidase protein. Deuterohemin gave a synthetic peroxidase with one mole hemin per mole protein that was active, though less active than the natural peroxidase (63%). Mesohemin gave 57% activity (see Fig. 4). One molecule of each hemin shown in the figure can combine with one molecule of protein. Hematohemin failed to give any activity with the protein. The later positive results of Gjessing and Sumner (31) are doubted by Theorell (80). This means that the 2- and 4-vinyl groups may be removed (deutero-), or may be saturated with hydrogen (meso-); however, replacement of these groups with hydroxyl and hydrogen (hemato-), respectively, abolishes the entire activity.

On the other hand, no changes in the propionyl groups in positions 6 and 7 seem to be compatible with the restoration of peroxidase activity. Pheophorbide a, chlorine e<sub>1</sub>,  $\gamma$ -phyllhemin, pyrrohemin, and rhodohemin

were inactive. Especially interesting are the latter two compounds: the pyrohemim differs from the mesohemim only in lacking the 6-propionyl group, and the rhodohemim differs from the mesohemim only in having the 6-propionyl group substituted by a carboxyl. One may conclude that acid groups are needed in both 6 and 7 positions, and that the length of the carbon chain plays a decisive role.

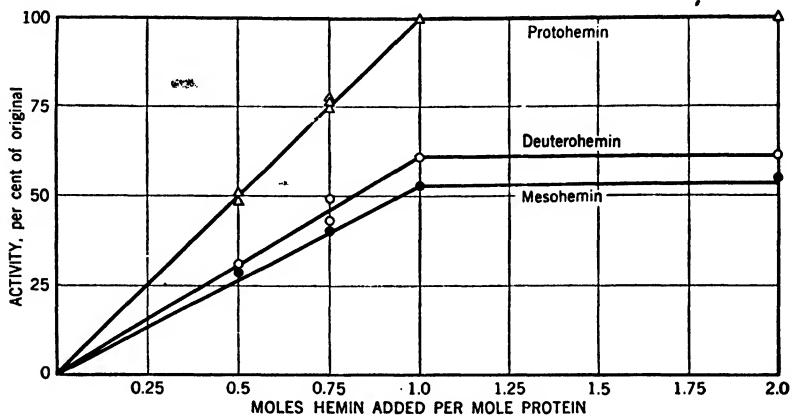


Fig. 4. Combination of peroxidase protein with proto-, meso-, and deuterohemins (86).

The attempts of Gjessing and Sumner (31) to reactive peroxidase protein by manganese protoporphyrin were criticized above (page 267).

#### B. SPECTROPHOTOMETRIC INVESTIGATIONS

The light absorption curve of pure horse-radish peroxidase is shown in Figure 5. The spectrum is similar to that of hemoglobin (47). The pure preparation of Theorell showed absorption maxima at 270, 402, 500, and 640  $m\mu$ , with a slight reinforcement at 550  $m\mu$  visible as a faint band in the spectroscop.

The so-called "paraperoxidase" or "peroxidase I" (71,76) is possibly a derivative of the peroxidase. It is a hemichrome, according to the absorption spectrum. Upon reduction in slightly alkaline solution it gives a hemochrome spectrum; on acidification the spectrum is reversibly converted to the spectrum of the ferroperoxidase. One may imagine it is formed by a deformation of the peroxidase protein to bring a suitable group into proximity to the iron, so that a loose bond can be formed. The activity of the paraperoxidase in the purpurogallin test is about the same as that of the peroxidase.

The preparations of Keilin and Mann probably contained a mixture of peroxidase and paraperoxidase.

The brown color of the peroxidase turns red in alkaline solution, and the absorption bands shift to 583 and 549  $m\mu$  (Keilin and Mann, 47). Theorell's (77) spectrophotometric determination of the dissociation constant gave the value 10.9; the magnetometric determination gave the value

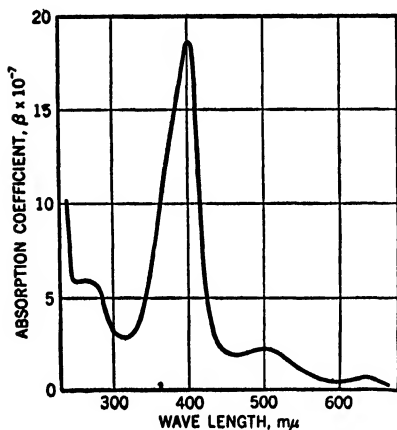


Fig. 5. Light-absorption curve of pure horse-radish peroxidase (73). Absorption maxima are at 270, 402, 500, and 640  $m\mu$ ; a slight reinforcement at 550  $m\mu$  was visible as a joint band in the spectroscope.

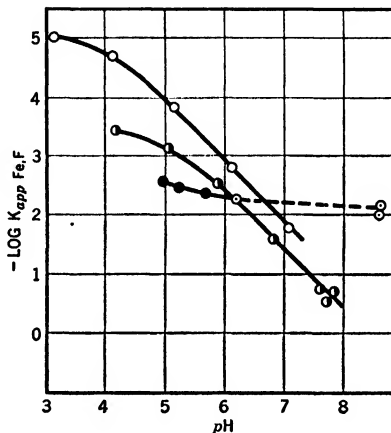


Fig. 6. Dissociation curves (87). Experimentally determined points for  $pK_{app}$  of horse-radish peroxidase fluoride (○) and of lactoperoxidase fluoride (○).  $pK_{Fe.F}$  in methemoglobin: Theorell and Paul (●); Coryell, Stitt, and Pauling (○).

11.27 (see page 299). The change looks spectroscopically similar to the shift hemoglobin  $\rightarrow$  hemoglobin hydroxide with  $pK$  of 8.1 (see page 275), but the magnetic determinations revealed a profound difference (see below).

Reduction of the peroxidase with hydrosulfite gives a red solution of ferroperoxidase with the bands 594.5 (narrow) and 558  $m\mu$  (broad and flat). On shaking with air the ferroperoxidase is reoxidized, apparently without the formation of an oxyferrous compound. It thus differs in this respect from hemoglobin. The ferroperoxidase gives a red carbon monoxide compound with two bands at 578 and 545.5  $m\mu$ . The intensity and size resemble the bands of carbon monoxide hemoglobin, but are displaced 8  $m\mu$  toward the red.

KCN, nitric oxide, and hydrogen sulfide produce red compounds with the ferriperoxidase with two bands each in yellow and green. Azide gives a similar compound, but only in acid solution. Fluoride gives a compound of another type, with an intensive band at 615  $m\mu$ , and other bands at 561, 530, and 496  $m\mu$  (47). The dissociation constants of the fluorides of horse-radish peroxidase and lactoperoxidase were studied spectrophotometrically at different  $pH$  values by Theorell and Paul (87). The results are shown in Figure 6. The change in light absorption at 615  $m\mu$  (for horse-radish peroxidase) and 620  $m\mu$  (for lactoperoxidase) on addition of increasing amounts of fluoride at constant  $pH$  gave theoretical dissociation curves with the  $pK$  values as indicated in Figure 6. The unbroken lines are calculated on the assumption that the fluoride ion competes with a dissociable hydroxyl group for the same co-ordination place at the iron. The formula:

$$K_{Fe,OH} = \frac{K_{Fe,F} \times [OH^-]}{K_{app} - K_{Fe,F}}$$

was deduced for this case. The theoretical values are as follows: for horse-radish peroxidase,  $K_{Fe,OH} = 10^{-9.0}$ ,  $K_{Fe,F} = 10^{-3.46}$ ; for lactoperoxidase,  $K_{Fe,OH} = 10^{-10.04}$ ,  $K_{Fe,F} = 10^{-5.1}$ .  $K_{Fe,OH}$  means the dissociation constant for hematin iron and hydroxyl and  $K_{Fe,F}$ , the same for fluoride and hematin iron without hydroxyl.  $K_{app}$  is the observed value which is a function of  $K_{Fe,OH}$ ,  $K_{Fe,F}$ , and  $[OH^-]$ . It will be seen that the curves, calculated on the assumption of the above formula and the dissociation constants indicated below the figure, fit exactly the values found experimentally. It can thus be said with certainty that the horse-radish peroxidase and the lactoperoxidases have one hydroxyl group dissociably attached to the iron. The  $pK$  for the former is 5.0, for the latter, 4.0.

Careful spectrophotometric measurements on horse-radish peroxidase at 655  $m\mu$  and different  $pH$  values revealed the existence of a heme-linked group with  $pK$  of 4.0. Its dissociation curve coincides with the activity measurements (purpurogallin tests) from  $pH$  7 to  $pH$  3, see Figure 7.

It seems to us to be of great interest that the enzyme activity is thus correlated to the dissociation of a single heme-linked group. The dissociation state of all the other, not heme-linked groups of the peroxidase molecule plays no role in the activity. Our theory emphasizing the predominant importance of heme-linked groups for the activity (see page 269) was verified in this case. Moreover, we find that not all heme-linked groups necessarily interfere with the activity; it is immaterial whether the hydroxyl group is attached to the iron or not.

Horse-radish peroxidase on addition of hydrogen peroxide may give three spectroscopically very different compounds. A small amount (one to two moles hydrogen peroxide per mole peroxidase) first gives a greenish compound ("I") (73,77) characterized by a sharp band in red at 655 to 658  $m\mu$ . This is spontaneously converted to a red compound, first found by Keilin and Mann (47), with bands at 530 and 560  $m\mu$ . Keilin and Mann numbered this compound "I," which we have called "II." A third red

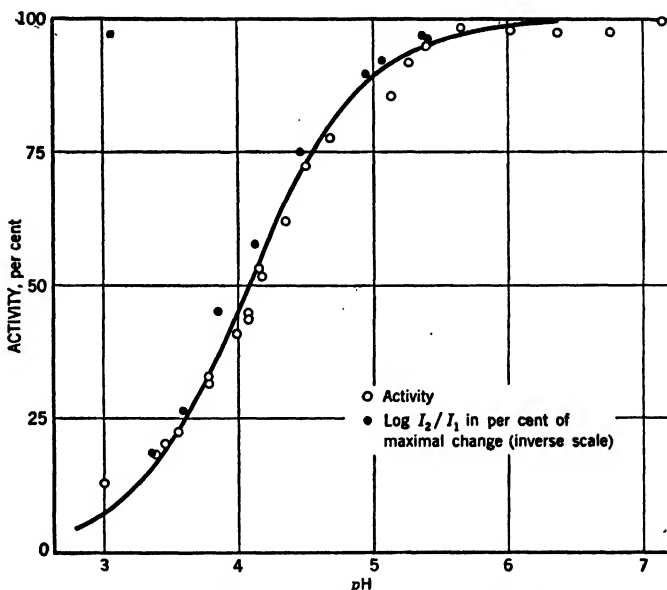


Fig. 7. Activity (O) in purpurogallin test and light-absorption change at 655  $m\mu$  (87).

compound is formed on addition of hydrogen peroxide in excess (ten to twenty moles per mole peroxidase). We think our "I" must be the physiologically important compound, since "II" and "III" (= Keilin and Mann's "II") hardly have time to appear during the rapid reaction cycle of peroxidase.

### C. MAGNETOMETRIC MEASUREMENTS

Magnetometric measurements were carried out with a micromethod by Theorell (75,77). The change in spectrum and color with  $pK$  10.9 is accompanied by a change in bond type from essentially ionic bonds,  $\chi_{mol} =$



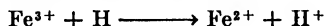
$12,560 \times 10^{-6}$  (same value as for acid hemiglobin, 22) to essentially covalent bonds. The magnetometric determination gave a  $pK$  of 11.27. The fluoride compound, just as in the case of hemiglobin fluoride, reached the theoretical value for five odd electrons,  $\chi_{mol} = 14,700 \times 10^{-6}$  corresponding to 5.92 Bohr magnetons. The cyanide compound was found to contain one cyanide per iron and to have the iron linked by covalent bonds; this was also the case with the hydrogen sulfide peroxidase.

A mixture of hydrogen peroxide peroxidases I and II gave intermediate values that may perhaps be interpreted as arising from ionic bonds in I and covalent bonds in II. Ferropoxidase had four odd electrons per iron atom (thus ionic bonds), whereas the carbon monoxide ferropoxidase was diamagnetic (covalent bonds).

Differential titration experiments were carried out on three systems (79). Ferropoxidase and carbon monoxide ferropoxidase gave identical curves. The peroxidase thus shows no Bohr effect. Since the two derivatives correspond as to bond types to the analogous hemoglobin and carbon monoxide hemoglobin, it can be stated with fair certainty that the heme-linked groups in horse-radish peroxidase are *not imidazole*.

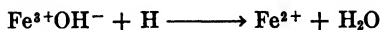
The titration difference between the ferri- and ferro-peroxidase was determined in the following way. Ferriperoxidase was titrated; another portion of the same solution was reduced with platinum and hydrogen after addition of one mole safranine per mole peroxidase as reduction mediator, since ferriperoxidase is not reduced by platinum and hydrogen alone.

One would, after correction for the titration value of leucosafranine, expect a difference of one equivalent per mole:



under the condition that the heme-linked groups and their state of dissociation are the same in the ferric and ferrous forms. This held true for the  $pH$  region from 8 to 9.5. The difference decreased from  $pH$  9.5 to 11.5 as was expected from the presence of the heme-linked group in ferriperoxidase with  $pK$  11. This group obviously corresponds to the one found spectrophotometrically and magnetometrically. This group is thus not detectable in ferropoxidase in this  $pH$  range. Below  $pH$  8 (unpublished) the difference decreased down to practically zero between  $pH$  4 and 5.5, indicating a dissociation constant of ferropoxidase in the neighborhood of  $pK$  7. This seemed hard to explain at the time; we did not know about the existence of a hydroxyl group bound to the iron in ferriperoxidase. Since we now have to assume that the hydroxyl group is split off on reduction, it is

easy to explain how the titration difference between ferric and ferrous can disappear:



On the other hand, we lack an explanation for the heme-linked group with  $pK$  7 in ferroperoxidase. It cannot be a hydroxyl bound to the ferrous iron, because such a hydroxyl should be displaced by carbon monoxide and appear in the titration of ferroperoxidase against carbon monoxide ferroperoxidase. This was not the case, and furthermore it seems hard to imagine how a hydroxyl group could be bound to ferroheme by ionic bonds.

*The titration difference between* the free protein component and resynthesized peroxidase was two equivalents from  $pH$  5.5 to 9. The difference in the region 9 to 11.4 indicated the presence of a titratable group in the protein with  $pK$  of 10; this group disappeared after resynthesis with protohematin, perhaps by coupling to one of its propionyl groups. It should be recalled in this connection that intact propionyl groups of the hematin are indispensable for the restoration of activity (page 288).

#### D. NATURE OF THE HEME-LINKED GROUPS IN PEROXIDASE

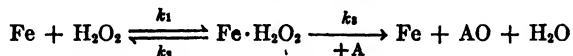
Summarizing the evidence that may be regarded as conclusive at present we find the following: (1) Horse-radish peroxidase has a hydroxyl group attached to the iron by essentially ionic bonds. Its  $pK$  is 5.0. Lactoperoxidase has a similar group of  $pK$  4.0. (2) The decrease in activity of horse-radish peroxidase in acid solution follows a simple dissociation curve with  $n = 1$ . This curve coincides with a small, reversible change of light absorption at  $655 m\mu$  which was determined spectrophotometrically. The dissociation of this unknown heme-linked group thus alone determines the degree of activity. (3) Horse-radish ferriperoxidase has a heme-linked group of  $pK$  11 that can be demonstrated magnetometrically (change to covalent bond type) and spectrophotometrically. Before the existence of the iron-linked hydroxyl group with  $pK$  was known it seemed reasonable to assume that the group with  $pK$  11 was a hydroxyl, analogous to the hydroxyl with  $pK$  in hemoglobin. Now it must be admitted to be unlikely that two hydroxyls could be bound (above  $pH$  11) to the same hematin iron, so the nature of the group with  $pK$  11 is still unknown. (4) The two propionyl groups of the hematin are indispensable for the resynthesis of an active horse-radish peroxidase from hematin and the protein component, whereas the vinyl groups can be saturated with hydrogen or even removed without losing the entire activity. There is some indication that one of the

propionyl groups is linked to a group that is titrated with a  $pK$  of about 10 in the free protein.

The data available so far are insufficient to permit us to draw conclusions concerning the nature of the protein group attached to the iron. It is possible that it could be a carboxyl group; this would seem less surprising since it has been found that the iron in catalases gives compounds with carboxylic acids (see below). But the interpretation of the differential titration data on horse-radish peroxidase and its derivatives became less clear after the discovery of the hydroxyl group with  $pK$  5. It is hoped that Paul's measurements of the redox potential, now in progress, will give additional evidence as to nature of the iron-linked protein group in peroxidase.

#### E. MODES OF ACTION OF PEROXIDASE

Britton Chance (16), using a modification of the Hartridge-Roughton flow method, studied the kinetics of the formation and dissociation of a primary peroxidase-hydrogen peroxide compound, and its reaction with acceptors like ascorbic acid and malachite green:



Values for the constants were found to be:  $k_1$ ,  $1.2 \pm 0.4 \times 10^4$  liter mole<sup>-1</sup> sec.<sup>-1</sup>;  $k_2$ ,  $< 0.2$  sec.<sup>-1</sup>; and  $k_3$  varies with the acceptor and its concentration from nearly zero to  $> 5$  sec.<sup>-1</sup>. The Michaelis constant by different methods was calculated to be  $0.41 \times 10^{-6}$  to  $0.50 \times 10^{-6}$ .

Chance, at the time his work was published, did not know about Theorell's green peroxidase-hydrogen peroxide I. There is no evidence, however, in Chance's experiments against our view that the primary reaction product, which is the physiologically important one, is this green compound with an absorption band at 655-658  $m\mu$ . By way of analogy we may presume that the iron is held by essentially ionic bonds in the compound as well as in the free ferriperoxidase. This could be the reason for the high velocity of the over-all enzyme reaction. With higher hydrogen peroxide concentrations, compound III is formed, in which the iron is very probably held by covalent bonds.

The reaction velocity of this compound is probably comparatively slow, since Sumner and Gjessing (66) found, for horse-radish peroxidase, that increasing the hydrogen peroxide concentration above an optimal concentration gave a successive decrease in the amount of purpurogallin formed. However, it was not established whether the decrease was due to the formation of compound III or to destruction of the enzyme.

Summarizing, it seems reasonable to ascribe the high velocity of peroxidase reactions to the ability of the peroxidase to form an ionic compound with hydrogen peroxide. Other hemoproteins, such as hemoglobin, seem to be unable to form analogous compounds; hemoglobin-hydrogen peroxide spectroscopically resembles peroxidase-hydrogen peroxide III.

The peroxidases, in the type of reactions described above, remain in the ferric state. They are for instance not inhibited by carbon monoxide. However, the peroxidases may function as *aerobic oxidases* on dihydroxymaleic acid (88). This reaction is inhibited by carbon monoxide, and the inhibition is reversed by light. This fact shows that a reversible oxidation-reduction of the peroxidase is involved in this case. Hydrogen peroxide must be formed in traces as a necessary intermediate product, since the oxygen uptake can be promptly stopped by the addition of catalase.

It is interesting to note that if dihydroxymaleic acid were present in some living cells—which owing to its instability is hard to establish—the peroxidases could exert a function analogous to Warburg's respiratory enzyme. The turnover number of the peroxidase iron in catalyzing the oxidation of dihydroxymaleic acid is of the order of magnitude  $10^5$  per minute, thus about the same as in the ordinary peroxidase reaction.

## V. Catalases

The earlier literature up to 1941 has been reviewed by Sumner in the first volume of this publication. For that reason much of the older evidence need not be repeated here.

As may be seen in Table I (page 266), several new catalases have been crystallized in the last years. The "active" group in catalases is protohematin (Zeile and Hellström, 94; Stern, 58). In addition to this some catalases contain a blue component which is split off by treatment with acetone and hydrochloric acid. Stern, who described the blue product first, believed it to be an impurity, whereas Sumner and Dounce (62) reported it to be a decomposition product of pure catalase. Lemberg, Norrie, and Legge (49) crystallized it and defined it as biliverdin.

The catalases from beef and horse blood (7,48) from human blood (13), and from horse kidney (14) are free of biliverdin. It can thus be said with certainty that the biliverdin component is immaterial for the catalase activity. Human liver catalase (14), surprisingly enough, is free of biliverdin.

Bonnicksen (13), using amino acid analyses and immunological tests, proved that the protein component of the horse blood and liver catalases are

identical. "The probability of a common origin to liver and blood catalase is favoured by the identity of the protein group of both substances. Perhaps liver catalase is nothing but blood catalase liberated from the red corpuscles during their destruction." It seems reasonable to assume that part of the catalase hematin could be oxidized to biliverdin in the liver.

Attempts to determine the nature of the heme-linked groups in catalases were met with greater difficulties than in any other kind of hemo-proteins. This is due to several unfavorable circumstances: catalase hematin cannot be reduced by ordinary reducing agents; catalases cannot be split reversibly; the interpretation of many different data is obscured by interaction phenomena between the four iron porphyrins in each molecule. Furthermore, until recently only liver catalases with two different kinds of colored groups were available. This last drawback is now overcome with the isolation of easily available catalases containing only hematin and protein.

For the reasons mentioned above, differential titration and redox potential determinations cannot be used to determine the nature of the heme-linked groups. Under such conditions it seems impossible to solve the whole question of the heme-linked groups of catalases by the aid of the methods we have at our immediate disposal. Some amino acid analyses have been carried out, but these cannot yet contribute to our knowledge of the heme-linked groups. However, some information has been obtained from magnetometric observations, from spectrophotometric studies of different catalase compounds with inhibitors, and from activity determinations in solutions of different *pH* values and varying concentrations of inhibiting anions.

#### A. AMINO ACID ANALYSES

Amino acid analyses of horse liver catalase have been carried out by Theorell and Åkeson (78) and by Bonnichsen (13), who compared these values with those obtained on horse blood catalase. Bonnichsen's results largely confirmed the earlier values of Theorell and Åkeson. Furthermore, he analyzed the catalase for glutamic and aspartic acids and cystine. Horse liver catalase, according to Bonnichsen, contains: 3.86% histidine, 8.9% arginine, 7.1% lysine, 10.3% glutamic acid, 16.5% aspartic acid, and 1.85% cystine (mean values). The values for the horse blood catalase were, within the limits of error, the same. This applies to the nitrogen distribution on the fractions humin, anodic, neutral, and cathodic nitrogen as well.

## B. MAGNETOMETRIC DETERMINATIONS

Magnetometric determinations were carried out on beef liver catalase by Michaelis and Granick (50), but their value for the paramagnetic susceptibility, 4.64 Bohr magnetons, corresponding to three odd electrons, could not be confirmed by Theorell and Agner (81), who obtained the theoretical value 5.92 Bohr magnetons (five odd electrons,  $\chi_{mol} = 14,800 \times 10^{-6}$ ). The magnetometric titration of horse liver catalase with hydrogen cyanide showed that the addition of three moles of hydrogen cyanide per mole of horse liver catalase caused the paramagnetic susceptibility to decrease to  $\chi_{mol} = 6,830 \times 10^{-6}$ . According to Agner (6) horse liver catalase contains three protohematin groups and one extra atom of iron per molecule. The value found can be most easily explained by assuming that hydrogen cyanide forms covalent compounds with the protohematin, but does not combine with the nonhematin iron (or forms an essentially ionic compound with it, which would seem unlikely).

An attempt was made by Theorell and Agner (81) to determine the valency of the protoporphyrin iron in the azide-superoxide-carbon monoxide compound of horse liver catalase described by Keilin and Hartree (41). The theory of these authors on the mechanism of the catalase decomposition of hydrogen peroxide is now largely founded on the spectroscopically plausible assumption that the protoporphyrin iron in this complicated compound is ferrous. The magnetic measurement values of Theorell and Agner on azide-peroxide-carbon monoxide catalase fitted *ferric* protoporphyrin best. Keilin and Hartree recently pointed out (44) that this evidence may be inconclusive owing to the possibility of a reoxidation and to formation of microbubbles of oxygen in the glass tube. These objections are probably not justified because (1) a reoxidation would have been easily detectable by the reappearance of the catalase band in red and (2) the appearance of microbubbles of oxygen would have caused a continuous and fast change in the readings.

Nevertheless, we are better able now to solve the problem with the biliverdin-free catalases: if the iron is ferrous in the compound, we would expect it to be diamagnetic; if it is ferric, it should be paramagnetic, with one unpaired electron, since the bonds must be essentially covalent in both cases. However, it may be possible to find still better methods than the magnetometric ones in this particular case, where the experimental difficulties are great.

The azide and fluoride compounds were both found to have the iron

linked by essentially ionic bonds, whereas the hydrogen sulfide catalase gave intermediate values that were open to different interpretations.

### C. DERIVATIVES AND ACTIVITY INHIBITION

Catalase hematin gives derivatives with several different chemicals. Spectroscopic investigations leading to this conclusion were carried out by Zeile and Hellström (hydrogen cyanide) (94), Stern (ethyl hydroperoxide) (57) and Keilin and Hartree (sodium fluoride, nitric oxide, sodium azide, ammonia, hydroxylamine, hydrazine, and sodium azide with peroxides) (40). Some of the compounds are greenish in color (for instance with sodium fluoride or azide and hydroxylamine) and have their iron linked by essentially ionic bonds; see above, others are reddish with bands in yellow and green and have their hematin iron attached by essentially covalent bonds (hydrogen cyanide, hydrogen sulfide, sodium azide with peroxide, nitric oxide, and ammonia). Recently Agner and Theorell (8) have shown that many or perhaps all anions are able to give greenish compounds with catalase iron and to cause at the same time an inhibition of the activity. Michaelis and Pechstein (51) already had found that sulfate, chloride, acetate, and nitrate ions cause an inhibition, and that a reciprocal relation existed between the hydrogen ion and the anion concentration.

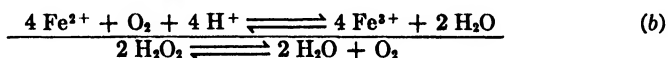
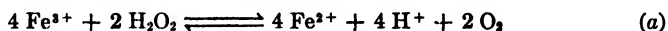
The determinations by Agner and Theorell of the changes in light absorption on addition of different anions (phosphate, acetate, formate) showed, in a way analogous to that described for the peroxidases, that a *hydroxyl group* is attached to the hematin iron, so that other anions have to compete with the hydroxyl for the bond to iron. This explains why the anion inhibition is increased with decreasing hydroxyl concentration (decreasing pH). The dissociation constant for catalase iron and hydroxyl was calculated as  $K_{\text{Fe},\text{OH}} = 10^{-10.2}$  ( $pK_1 = 3.8$ ). It is thus of the same order of magnitude as in lactoperoxidase. The dissociation constant  $K_{\text{Fe},\text{Ac}}$  was calculated to be  $10^{-2.5}$ , whereas the formate had a much higher affinity for catalase iron,  $K = 10^{-6.4}$ . This means, for example, that at pH 5, 0.0001 *N* formate will cause 50% inhibition.

As all investigated anions (phosphate, chloride, sulfate, fluoride, formate, acetate, lactate, propionate, and hippurate) in sufficiently high concentration and at low pH were found to inhibit catalase by forming compounds, it proved a problem to acidify a catalase solution without causing an anion effect at the same time. However, the compound with hippurate showed high enough dissociation to permit acidification of diluted catalase solutions with hippuric acid without causing any anion in-

hibition. In such solutions the catalase activity was much less decreased in acid solution than earlier authors had found with buffer solutions, containing inhibiting anions. At pH 3.3, the activity was still over 50% of the value at pH 6.8. Possibly the hydroxyl group is necessary for activity, though this could not be ascertained definitely.

#### D. MECHANISM OF CATALASE ACTION

In 1938 Keilin and Hartree (42) proposed the following scheme for the catalase reaction with hydrogen peroxide:



The theory was based mainly upon two kinds of evidence: (1) It was stated that catalase activity was decreased in pure nitrogen, by removing the oxygen in equation b. Various authors (38,61,81,90) were unable to confirm these experiments, and Keilin and Hartree (43) themselves revealed that the inhibition found in their experiments was caused by nitrogen oxide impurities. (2) Addition of peroxides to azide catalase gave a red compound with bands at 590 and 554 m $\mu$ . In carbon monoxide these bands shifted to 580 and 545 m $\mu$ . This was explained by assuming that hydrogen peroxide would reduce the azide catalase to the ferrous state. The compound could be restored to azide catalase by shaking with oxygen, but potassium ferricyanide failed to do the same. On the other hand, sodium thiosulfate caused no change either. The magnetic measurements of Theorell and Agner seemed to favor the presence of ferric iron, but this evidence was questioned by Keilin and Hartree (44), who presented new experiments to support their theory. They measured catalase activity on hydrogen peroxide in carbon monoxide-nitrogen carbon and monoxide-oxygen mixtures in the presence of azide in concentrations which were in themselves too low to have marked inhibitory effect. Their results give strong support to the view that *azide catalase* can be reduced by hydrogen peroxide to a compound that combines with carbon monoxide. But the authors seem to overlook that their experiments give strong evidence against the validity of their theory for catalase without azide: "Under these conditions azide-free catalase was inhibited neither by N<sub>2</sub> nor by CO, when compared with air as standard." Their curve (Fig. 8) clearly shows that carbon monoxide inhibition is conditioned by the presence of azide.

We suggested in 1942 (81) that the reaction mechanism may be pro-



foundly changed by azide. It was pointed out then that peroxidases with hydrogen peroxide and pyrogallol give a reaction that is not inhibited by carbon monoxide because the iron remains ferric, whereas their reaction with dihydroxymaleic acid shows a light-sensitive carbon monoxide inhibition. Therefore it seems unjustified to assume similar reaction mechanisms for azide catalase and free catalase, as the former is inhibited by carbon monoxide, while the latter is not.

**Coupled Oxidations.** In a recent paper Keilin and Hartree (46) report an interesting set of experiments presenting additional evidence in favor of their previous assumption (39) that catalases function physiologically as secondary oxidation catalysts. It has been fairly generally assumed by other authors that the function of the catalases was to remove hydrogen peroxide from the tissues in order to protect sensitive substances, for instance hemoglobin, from being destroyed by oxidation. Keilin and Hartree now were able to demonstrate coupled catalase oxidations of methanol, ethanol, *n*-propanol, isobutanol,  $\beta$ -aminoethanol, and ethylene glycol. The peroxide was formed by primary oxidation systems such as xanthine oxidase and acetaldehyde or glucose oxidase and glucose. The catalase then uses the hydrogen peroxide for oxidizing the alcohols to aldehydes.

Chance (16a) recently demonstrated the formation of an intermediate compound of catalases and hydrogen peroxide. The compound had many properties in common with the intermediate compound of hydrogen peroxide and peroxidase. The spectrum could be determined in the region of the Soret band showing a small shift of the band toward the red. The rate of formation of this compound was  $3 \times 10^7$  liter mole<sup>-1</sup> sec<sup>-1</sup>, exceeding the value required by the Michaelis theory for catalase activity. Without the addition of acceptor the compound decomposed slowly at about the same rate as the peroxidase-peroxide compound. The catalase activity increases the equilibrium constant to  $1 \times 10^{-6}$  mole liter<sup>-1</sup>. The inter-

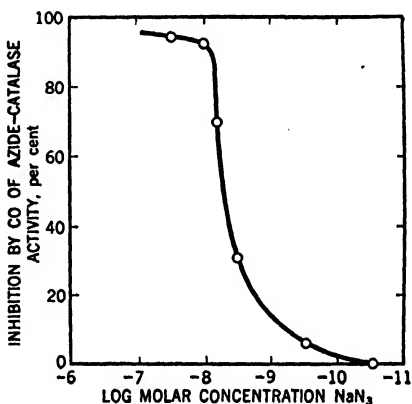


Fig. 8. Effect of azide on carbon monoxide inhibition (46). Catalase hematin =  $1.86 \times 10^{-8}$  M.

mediate compound reacts rapidly with lower alcohols and with ascorbic acid. By comparing the kinetic data with Keilin's experiments, the intermediate compound of Chance was proved to be the cause of the "coupled oxidations" mentioned above. It is thus obvious that "coupled oxidation" is nothing but a true peroxidase reaction of catalases. The only physiological difference between peroxidases and catalases thus would be that catalases decompose any excess of hydrogen peroxide—and this excess may never be present in the tissues—whereas peroxidases do not.

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# DISTRIBUTION, STRUCTURE, AND PROPERTIES OF THE TETRAPYRROLES

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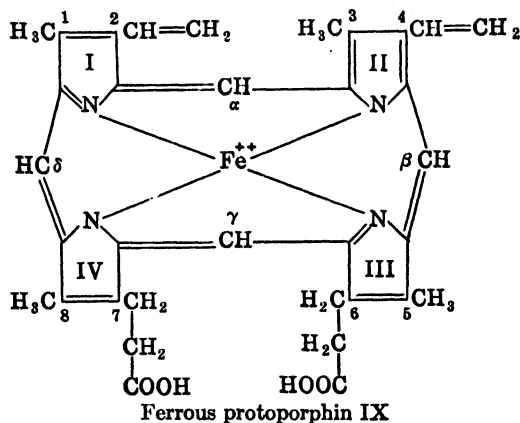
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## I. Introduction

Although great progress has been made in the elucidation of the chemical structure of the tetrapyrroles and in the study of their activities, a large gap still remains in our understanding of the pathways and mechanisms of their biological synthesis. It is with the idea of developing an approach to a study of tetrapyrrole synthesis that this review has been written. No attempt has been made to be complete but rather we have attempted to point out and illustrate those general ideas which appear as unifying concepts. For our knowledge of the fundamental chemical structure of the tetrapyrroles we are indebted to a number of the illustrious in organic chemistry, among them Hans Fischer, Willstätter, Küster, Nencki, and Piloty.

By way of introduction, we shall review a few of the properties of one of the most important representatives of the tetrapyrroles, protoporphin IX,\* which is present in nearly all cells.



Protoporphin IX is made up of four pyrrole rings (I, II, III, and IV) which are attached to each other through four  $\equiv\text{CH}$ , methene, bridges ( $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ). The inner sixteen-membered ring is formed of alternating single and double bonds. Such a ring may be considered as representing, to a limited degree, a circuit for the conduction of the mobile  $\pi$  electrons which are responsible for the resonating structure. A number of

\* The following terminology with respect to the iron porphin complexes will be used: porphin for porphyrin, heme or iron porphin for ferrous or ferric porphin; hemo-chromogen for ferrous or ferric porphin co-ordinating with two basic nitrogen-containing atoms or atom groups.

properties derive from this resonating structure. The compound acquires an "aromatic" character, like benzene. It becomes greatly stabilized and can be halogenated, nitrated, and sulfonated without being split into fragments. Its reduction potential is lowered to such an extent that, unlike most dyestuffs, it is not reduced by hydrosulfite or hydrogen-palladium in alkaline solution, but requires sodium amalgam for reduction. The individual pyrrole rings, which by themselves are quite reactive, and tend to polymerize to resins, are also stabilized by forming a portion of the resonating porphyrin structure. Their stability is further enhanced by the presence of side chains on the outer ( $\beta, \beta'$ ) carbon atoms of the pyrrole rings. The side chain pattern of protoporphyrin IX around the ring is: methyl, 1; vinyl, 2; methyl, 3; vinyl, 4; methyl, 5; propionic acid, 6; propionic acid, 7; and methyl, 8. The resonating porphyrin has a flat structure, all of the resonating atoms lying in the same plane. Because of resonance, light absorption is greatly enhanced and shifted to longer wave lengths, the absorption spectrum consisting of a series of prominent bands extending through the visible region. When ferrous iron is inserted into the ring, ferrous protoporphyrin IX is formed.

## II. Distribution, Structure, and Function of Naturally Occurring Tetrapyrroles

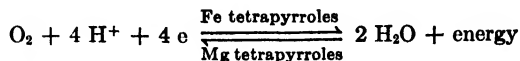
### A. GENERAL SCHEME OF THEIR FUNCTION

It is only when the porphyrins form metal co-ordination compounds that they appear to develop properties of great physiological significance. Of all the metals which can be introduced chemically into the ring only two are of importance biologically, namely, the iron porphyrins or heme compounds, and the magnesium phorbins or chlorophylls. In protoplasm the basic energy-yielding reaction may be considered to be the combination of protons and electrons with oxygen to form water, and the basic energy-storing reaction to be the reverse of this.\* This energy-yielding and energy-storing process is brought about by tetrapyrrole compounds. Thus the iron tetrapyrroles are utilized for the release of energy by activating oxygen to combine with electrons and protons, to form water; the magnesium tetrapyrroles appear to be utilized essentially for the storage of the energy of sunlight by catalyzing the removal of oxygen from water

\* The electrons are not free but are bound in some organic substances of higher potential energy. When the electrons are combined into the compound, water, they are at a lower potential energy.



with the release of electrons and protons (145). These reactions are summarized by:



If we assume with Oparin (97) that the earth's atmosphere was originally devoid of oxygen, then one might suppose that chlorophyll brought about the accumulation of oxygen and that chlorophyll preceded, or was formed simultaneously with, heme.

#### B. THE IRON PORPHINS (HEMES)

**Occurrence.** The function of the iron porphins, when associated with particular proteins, is to make oxygen available to the cell, oxygen being the ultimate electron acceptor in protoplasmic processes. Hemes, as components of the oxygen-activating enzymes including the cytochromes, are found in all cells that have been examined, with the exception of certain anaerobic bacteria. Even in the invertebrates in which the copper protein, hemocyanin, is used for oxygen transport, the oxygen activators of the tissue cells are proteins containing hemes (10).

Bacteria possess more or less complete cytochrome systems, depending on their degree of aerobiosis. Thus the true aerobes contain most of the cytochrome components, facultative anaerobes have an incomplete cytochrome spectrum, and the anaerobes have none. These bacterial cells which are said to lack hemes are of interest from the viewpoint of the origin of cell organization. Might these bacteria, lacking heme, represent primitive cells, organized during a period in which oxygen was not yet present in the atmosphere? Or, are they derived from cells which originally produced heme but have lost this function? Certain evidence indicates that these bacteria have lost the ability to synthesize this compound. Topley and Wilson (139) note that the obligate anaerobes *Clostridium sporogenes* and *Cl. histolyticus* actually contain small amounts of catalase, *i.e.*, a heme enzyme. If this is so, it may be considered evidence that heme is present in these cells.

Recently Gilder (50) found that *Leuconostoc mesenteroides*, which is a facultative anaerobe, when grown in the absence of iron protoporphin contains no catalase, but that catalase activity is present when the organism is grown in the presence of this substance. This experiment suggests that the organism has lost the ability to synthesize heme, yet still retains the ability, in a limited degree, to produce the specific apoprotein (*i.e.*, enzyme minus the prosthetic group, heme) of catalase. The addition of

iron protoporphin to *Leuconostoc* does not immediately bring about the production of catalase, indicating that the apoprotein of catalase is not present in the cells to any appreciable extent. Therefore one must assume either a continuous synthesis and destruction of the apoenzyme, little being present at any one time unless stabilized by attaching to iron protoporphin, or else a stimulation to produce the apoenzyme in the presence of the iron protoporphin. Thus cells which had been considered to be examples of cells lacking heme enzymes are found to contain heme enzymes or vestiges of them. It becomes less likely, therefore, that primitive cells, *e.g.*, bacteria, will be found in which the mechanism for heme synthesis had never developed.

#### **Factors Effecting Specialization of the Function of Iron Porphins.**

Inorganic iron salts may be looked upon as evolutionary precursors of the iron porphins. Depending on the *pH* of the medium, they possess the properties of an oxidase and of an electron transporter, but their ability to function as a catalase or peroxidase is very slight. When iron is incorporated into the porphin ring, the catalase and peroxidase activities are enhanced. When the iron porphins become attached to specific proteins, not only do these particular protein compounds acquire marked degrees of specialization and enormous enhancement of activity, but the hemes are also stabilized and protected. For example, comparing catalase activity, iron porphin is  $10^3$  times more active than iron salts, and catalase is  $10^9$  times more active (82,127). Depending on the protein to which it is attached, heme may function as transporter of molecular oxygen, transporter of electrons, activator of oxygen, activator of hydrogen peroxide, and decomposer of hydrogen peroxide (127).

How are this diversity of properties, marked degree of specialization, and enhanced activity brought about? A few mechanisms are already evident which may in part explain some of these phenomena.

The properties of the heme proteins are already present in a primitive fashion in ferrous and ferric iron itself. In its ionic state iron has a tendency to form octahedral complexes with six co-ordinate bonds. The co-ordination, in general, appears to be with oxygen or nitrogen groups containing unshared electron pairs, as it is in the heme compounds. Ionic iron in acid solution or the ionic iron-hexacyanide complexes in neutral solution, are model electron transport substances, being reversibly oxidized and reduced with the exchange of single electrons. An example of primitive oxidase activity is the oxidation of ferrous hydroxide by molecular oxygen to the ferric state at *pH* 6-7, in the presence of a reductant which

may itself not be readily autoxidizable, for example, thioglycolic acid; the ferric iron as a thioglycolic acid complex may be reduced to the ferrous state again. The ferrous iron is now ready to be autoxidized again, and the cycle is repeated at the expense of thioglycolic acid, which may be considered to be catalytically oxidized to dithioglycolic acid. For details of this catalytic mechanism the reader is referred to the classic work of Michaelis and Schubert (94a). An interesting diversity of iron compounds with varying catalase or peroxidase properties of low degree may arise merely by the interaction of water with ferric ions at neutral pH. These hydrous ferric oxides are octahedral complexes and have been shown to vary in bonding, from a completely ionic type to a completely covalent type (94).

The porphins by themselves show no catalytic activities. When iron is incorporated into a porphrin it co-ordinates with four pyrrole nitrogen atoms lying in the plane of the porphrin ring, and may co-ordinate with two more atoms, one above and one below the plane of the ring. The iron porphins may co-ordinate either with two oxygen-containing groups, with two nitrogen-containing groups, or with one of each. Co-ordination with two oxygen groups may be illustrated by the compound ferric porphrin, which co-ordinates in alkaline solution (pH 10) with a hydroxyl group and a water molecule at the fifth and sixth co-ordination places (17), or by the compound ferrous porphrin  $\cdot \text{H}_2\text{O} \cdot \text{CO}$  (63a). Illustrations of iron porphins co-ordinating with one nitrogen group and one oxygen group are ferric porphrin  $\cdot \text{H}_2\text{O} \cdot \text{CN}^-$  and ferrous porphrin  $\cdot \text{pyridine} \cdot \text{CO}$ . When two nitrogen-containing groups are co-ordinated, one above, and one below the plane of the ring, an important group of compounds is produced, called the "hemochromogens" (6). For example, ferrous porphrin in water may be considered to co-ordinate with two molecules of water. If neutral nitrogen-containing compounds, such as pyridine, nicotine, or ammonia, are added to the solution, they will compete readily with the two water molecules for these co-ordination places.

An examination of the available data indicates that there is a tendency in solution for iron porphrin, whether ferrous or ferric, to form a hexavalent complex which is neutral as a unit, *i.e.*, bears a zero formal charge. If this correlation can be supported by further work it should prove a useful rule in analyzing for the co-ordinating protein groups of the heme enzymes. For example, neutral ferrous porphrin appears to co-ordinate with two neutral groups represented by either oxygen or nitrogen or both. Neutral oxygen atom groups of this kind are:  $\text{H}_2\text{O}$ ,  $\text{CO}$ ,  $\text{O}_2$ ,  $(\text{CO}_2?)$ ; and

neutral nitrogen atom groups are:  $\text{NH}_3$ , pyridine, imidazole,  $\text{HN}_3$ ,  $\text{NH}_2\text{-NH}_2$ ,  $\text{NH}_2\text{-OH}$ ,  $\text{HCN}$ ,  $\text{NO}$ ,  $\text{CH}_3\text{-NC}$ , ( $\text{N}_2$ ?). On the other hand, the ferric porphin, bearing a unit positive formal charge, appears to co-ordinate with two groups, only one of which is neutral. The other may be represented by oxygen atom groups bearing a unit negative formal charge as  $\text{OH}^-$ ,  $\text{HCOO}^-$ , (hydrogen peroxide as  $\text{HO}_2^-$ ?), or with nitrogen atom groups bearing a negative formal charge as  $\text{N}_3^-$  and  $\text{CN}^-$ . (In this group co-ordinating with ferric porphin, we may also include the negative ions,  $\text{F}^-$ , and  $\text{HS}^-$ .)

When a particular substrate is not present, for example, when hydrogen peroxide is not present in a catalase solution, the sixth co-ordination group may be occupied by a hydroxyl ion. If formate or acetate is present in the solution, it may replace the hydroxyl ion. The relative affinities of these various negative ions in competition with hydrogen peroxide for the sixth co-ordination place is suggested by Agner and Theorell (3) as being responsible for the apparent inhibition of catalase activity in the presence of these negative ions.

The hemochromogens, for example, dipyrindine heme, are thermodynamically reversible, univalent redox systems with a negligible activation energy for the acceptance of an electron in the oxidized state and the release of an electron in the reduced state (12). By varying the N compounds one may vary the redox level of the hemochromogen (11). If the nitrogen compounds co-ordinate more firmly with the ferrous form than with the ferric form, a substance of higher redox potential is produced (*e.g.*, dipyrindine heme). If the nitrogen compound co-ordinates more firmly with the ferric than with the ferrous form, a substance of lower redox potential is produced (*e.g.*, dicyanide heme) (17). Iron porphin itself is not a readily reversible system, perhaps because of its insolubility and tendency to polymerize. Its apparent redox potential is quite negative. The ability of the iron in the porphin to attach nitrogen compounds at the fifth and sixth co-ordination places would theoretically make it possible for the iron to attach to different nitrogen atoms in a protein molecule, and thus to give rise to a number of univalent electron-transferring substances of different redox potential.

Attachment of a particular nitrogen-containing group at the fifth co-ordination place may change the properties of the complex not only with respect to the redox potential but also with respect to the selective affinity for attaching a particular group at the sixth co-ordination place (27) (*e.g.*, ferrous heme·2 pyridine has a greater affinity for CO than

has the iron of free heme, the ferrous heme·pyridine·CO being a relatively stable complex). The variation in the affinity of heme proteins for different substrate molecules is most readily explained on this basis.

In ferrous hemoglobin there is some evidence that the fifth co-ordination link attaches to an imidazole nitrogen of the globin molecule (110). In the absence of molecular oxygen, the sixth co-ordination link may hold a molecule of water or another imidazole nitrogen loosely, depending on the pH of the medium (20). Magnetic susceptibility studies by Pauling and Coryell have further extended our knowledge of this molecule (100). When oxygen attaches at the sixth co-ordination place, a profound change in electronic configuration occurs. Not only do the two unpaired electrons of the oxygen become paired, but the four unpaired electrons of the ferrous iron also pair. This change in some way prevents the oxygen molecule from accepting an electron. If this stabilizing effect were destroyed by denaturing the globin and breaking the specific iron-imidazole link, an electron would immediately transfer from the ferrous iron to the oxygen molecule, and this denatured heme protein would then possess one of the properties of an oxidase enzyme, that is of being oxidized by molecular oxygen.

Not only do the proteins provide the selected atoms for co-ordination of the iron atom, but they also provide further means of anchoring the heme to the protein. An important type of attachment is by way of the propionic acid groups of the porphin, which adhere by coulomb forces to the protein (54,134). In the case of the hemochromogen, cytochrome c, an additional stabilizing factor is present; there is a direct chemical thio ether linkage through the vinyl groups to the cysteine of the cytochrome apoprotein; this constrains the two nitrogen atoms of the apoprotein to lie in the correct spacial configuration for co-ordination of the iron, in both the ferrous and the ferric states. Theorell has pictured the prosthetic group as lying in a crevice in the protein.

As will be discussed on page 333, the porphin ring at the methene carbon atoms is especially vulnerable to attack by hydrogen peroxide. One method for protecting the ring would be to bury the heme under a protective covering of protein. Such a protection would be especially desirable in the case of peroxidase and catalase. Also, the specificity of the enzyme would be enhanced by sterically hindering the approach of some larger groups to the sixth co-ordination place. Theorell and Paul (136) have shown that peroxidase may be split reversibly into the apoprotein and the heme, and may recombine to give the active peroxidase. However, unlike

the dissociation and recombination of hemoglobin, which is instantaneous, this process is quite slow with peroxidase, occurring within a period of minutes. Such a slow process might be due to a sterically hindered position in the apoprotein into and out of which the heme must adjust itself. It is interesting to note that it has not been possible to reversibly dissociate catalase, perhaps because it might be too well buried below the protein surface to diffuse out.

**Survey of Iron Porphin Proteins.** Of the different functions of the iron porphyrin compounds one of the best studied has been that of oxygen transport. Hemoglobin containing four heme groups per globin molecule is found widespread among the vertebrates in the red blood cells. In red muscle a muscle hemoglobin or myoglobin is found, in which only one heme is present per molecule. In invertebrates the oxygen-carrying proteins have a random distribution throughout various phyla, and differ from the proteins of vertebrates in size and composition (Table I). The hemoglobins of low molecular weight are contained in cells, whereas the hemoglobins of high molecular weight are found in hemolymph fluid. The porphyrin making up the prosthetic group in all the cases that have been studied is protoporphyrin. In the greenish oxygen-carrying protein spirographin, occurring in *Spirographis* and several other invertebrates, the prosthetic group is similar to protoporphyrin except that one vinyl group in protoporphyrin is replaced by a formyl group. One instance of hemoglobin in plants has been discovered by Kubo (81) and confirmed by Keilin and Wang (74). The hemoglobin is present in legume root nodules where nitrogen fixation goes on in the presence of *Rhizobium*. Curiously enough, neither the nitrogen-fixing bacteria, nor the plant roots separately, are able to produce hemoglobin. It is their symbiosis which leads to hemoglobin formation and nitrogen fixation in the root nodules. Virtanen and Laine (147) have shown that, when the legume plants are placed in the dark, the pink oxyhemoglobin turns to a brownish ferric hemoglobin. Some free heme also arises from the denaturing of the hemoglobin and finally the greenish bile pigment precursor, the so-called verdoglobin, is formed so that decomposition of hemoglobin appears to take the same pathway as in higher animals.

It is interesting to note here that, although these hemoglobins vary in molecular weight, amino acid composition, immunological properties, crystal structure, solubilities, etc., they all possess the common feature of binding oxygen reversibly to the iron of the porphyrin. One may surmise from these facts that all of the proteins may have in common on their surfaces



TABLE I (continued)

B. Porphins					
(?)	Coproporphin III Coproporphin I Uroporphin I	•	$(-\text{CH}_3)_4$ $1,3,5,7-(\text{CH}_3)_8$ $8-(\text{CH}_2\text{CH}_2\text{COOH})_4$ $1,3,5,7-(\text{CH}_2\text{CH}_2\text{COOH})_8$	$(-\text{CH}_2\text{CH}_2\text{COOH})_4$ $(-\text{CH}_2\text{CH}_2\text{COOH})_8$ $(-\text{CH}_2\text{CH}_2\text{COOH})_4$ $(-\text{CH}_2\text{CH}_2\text{COOH})_8$	(44,156)
C. Metal Porphins (Not Magnesium or Iron)					
(?)	Copper coporoporphin Copper uroporphin I Zinc coporoporphin I and III Vanadium porphin	Yeast Turquoise Acute porphyria Oil shales			(156) (140)
D. Magnesium Porphins					
Photosynthesis and oxygen production	Protochlorophyll a	Precursor of chlorophyll (?)	Magnesium porphin		(41)
	Chlorophyll a	Green plants	Magnesium dilydroporphin		(?)
	Chlorophyll b	Green plants	Magnesium dilydroporphin	$2-(\text{CH}=\text{CH}_2)$ $4-(\text{C}_6\text{H}_5)$ $2-(\text{CH}=\text{CH}_2)$ $4-(\text{C}_6\text{H}_5)$	(?)
	Chlorophyll c	Brown algae	Magnesium dilydroporphin (?)		(92)
	Chlorophyll d	Red algae	Magnesium dilydroporphin (?)		(128)
	Bacteriochlorophyll	Thiorhodase and sthiorhodase	Magnesium tetrahydroporphin	$2-(\text{COCH}_3)$ $4-(\text{C}_6\text{H}_5)$ $(\text{CH}_3)_4$	(?) (39)
E. Accessory Algal Protein Pigments					
Accessory pigments in photosynthesis (?)	Phycocerythrin	Red and blue-green algae	Tetrapyrrole related to protoporphin (?)		290,000 (83,119)
	Phycocyanin	Red and blue-green algae	Tetrapyrrole		275,000 (128b)
F. Open-Chain Tetrapyrroles					
Excretion products	Biliverdin Bilirubin Urobilin		$(-\text{CH}_3)_4$ $(-\text{CH}=\text{CH}_2)$ $(-\text{CH}_3)_4$	$(-\text{CH}_2\text{CH}_2\text{COOH})_4$ $(-\text{CH}_2\text{CH}_2\text{COOH})_8$ $(-\text{CH}_2\text{CH}_2\text{COOH})_8$	(119)

\* The prosthetic groups designated merely as "iron protoporphin" without "IX" indicate that the position of the side chains around the ring has not yet been established.



certain specific groups which are in the correct spatial position for binding the porphin and endowing the iron with this special oxygen-transporting property.

The enzyme catalase is present in almost all cells and has the function of splitting hydrogen peroxide into oxygen and water. Its prosthetic group is iron protoporphin IX (125) and was the first prosthetic group of the enzymes to be discovered (160). Recently Keilin and Hartree (73) reported that ethyl alcohol and a few other substances undergo oxidation in the presence of hydrogen peroxide and catalase, suggesting that catalase also has the function of a specific peroxidase.

Peroxidase, another iron protoporphin enzyme, activates hydrogen peroxide so that it becomes a powerful oxidizing agent. It has been crystallized by Theorell (133) from horse-radish and is also found in many plant and animal cells. A peroxidase specific for the oxidation of cytochrome c by hydrogen peroxide has been found in yeast (1).

The cytochromes found in most plant and animal cells consist of a number of iron porphin enzymes, distinguishable by their absorption bands. They are important because they make available to the cells about 90% or more of the oxygen that is used in respiration. Some of the cytochromes such as  $a_1$  and  $a_3$  are oxidases and function as activators of atmospheric oxygen, converting it to a powerful electron acceptor. Certain of the other cytochromes, like  $b_2$  and c, are electron transporters and act as intermediaries, shuttling electrons from the substrate to the oxygen-activating catalysts. Only one cytochrome, namely c, has been isolated in a pure form. Its prosthetic group may be looked upon as derived from iron protoporphin IX. Each vinyl group is modified in that a sulfur atom of cysteine from the protein adds to one carbon atom of the vinyl group and a hydrogen atom adds to the other. In general it may be surmised from their absorption spectra that the prosthetic groups of the cytochromes differ very little from iron protoporphin. Most animal and plant cells (51a) contain the cytochrome c-cytochrome oxidase system. Other cells, like *Escherichia coli* do not contain this system, and oxygen is made available through some other cytochrome pigments. Keilin and Hartree (72) have suggested that cytochrome oxidase might be identical with cytochrome  $a_3$  which appears to contain as its prosthetic group a substance related to iron spirographin. Haas (58) claims to have separated cytochrome oxidase into two components, a heat-labile, sedimentable fraction, and a heat-stable soluble fraction, neither of which by itself possesses significant activity. A new cytochrome, f, in plants has been reported by Hill (64), present

in  $1/150$  the concentration of chlorophyll, and with a potential above that of cytochrome c.

**Other Iron Porphins.** There are other, less well-defined systems in which iron porphins appear to play a part. Often the evidence presented is that some reaction is blocked by low concentrations of cyanide or by carbon monoxide. If the carbon monoxide complex is partially dissociable by light, the probability is greater that an iron porphin compound is involved. Only a few examples will be given by way of illustration. One of the most interesting of these is the hydrogenase reaction or one that is closely linked with it. Hoberman and Rittenberg (66) have shown that an enzyme system is present in *Proteus vulgaris* which catalyzes the exchange of hydrogen molecules or deuterium molecules with the hydrogen of water molecules. This reaction is inhibited by carbon monoxide and partially reversed by light; it is also inhibited by hydrogen cyanide, and peculiarly enough by oxygen, so that it is assumed that a heme catalyst in the system is active in the ferrous but not in the ferric state.

The nitrogen-fixing mechanism is also very sensitive to carbon monoxide. In the root nodules of red clover it is inhibited by as little as  $1 \times 10^{-4}$  to  $1 \times 10^{-3}$  atmospheres of carbon monoxide, and the nitrogen fixation in *Azotobacter* is inhibited by ten times this concentration, suggesting here also that heme is directly or indirectly involved (89).

In *Hemophilus influenzae* there is evidence that an iron porphin containing vinyl groups is needed for the reduction of nitrate to nitrite (54), and Quastel has reported that nitrate reduction by bacteria is affected by carbon monoxide (103a).

Several investigators have observed that the growth and course of the metabolism of some anaerobic bacteria is markedly influenced by the concentration of iron in the medium. Using the facultative anaerobe *Aerobacter indologenes*, Waring and Werkman (153) found that, on media containing little iron, catalase, peroxidase, some cytochromes, formic dehydrogenase, hydrogenase, and hydrogenlyase were very low or absent and that these appeared when the iron concentration was increased. The appearance of the heme enzymes indicated that as the iron was increased more heme was synthesized. Glucose fermentation on a low iron medium resulted in formic and lactic acids as the main end products, but in an iron-containing medium carbon dioxide and hydrogen were produced. They suggested that hydrogenlyase which decomposes formic acid to carbon dioxide and hydrogen was probably a mixture of three enzymes. One of them was postulated to be an iron-containing electron transporter, possibly a heme enzyme, which

might serve to mediate between the formic dehydrogenase component and the hydrogenase component.

*Clostridium* grown on low iron ( $7 \times 10^{-6} M$ ) produced lactate, formate, and ethanol, but, as the iron was increased ( $8 \times 10^{-5} M$ ), production of hydrogen and carbon dioxide was observed (61). This confirms the work of Pappenheimer and Shaskan (99) who, however, felt that some loose iron complex which might be disrupted by  $\alpha, \alpha'$ -bipyridine was the active catalyst rather than a heme enzyme.

That an iron porphyrin enzyme may have an essential function apart from its activation of oxygen or oxygen compounds is supported by the fact that the Turner <sup>1955</sup> strain of influenza bacillus, when grown under strictly anaerobic conditions, still requires heme for its growth (50).

### C. DISTRIBUTION OF THE PORPHINS

**Protoporphin IX.** As precursor of the most widespread iron porphins in nature, protoporphin IX, obviously, is itself found widely dispersed, although in minute amounts. It is possible to detect traces of a porphyrin by observing its red fluorescence when a tissue is illuminated with ultraviolet light (around 400 m $\mu$ ).

The red fluorescent substance in normal erythrocytes has been isolated and identified by Grotelpass (56) as protoporphin IX. It exists in blood in a concentration of only 15–40  $\gamma$  per 100 cc. of red cells. Reticulocytes are richer in it, and the immature red blood cells of the bone marrow, the normoblasts, contain still more. Protoporphin of the red cells is increased in cases of iron deficiency (some 10–20 times), in lead poisoning (some 40 times) (116), and in hemolytic anemia (155).

Protoporphin is deposited in the various bird egg shells at the time the shell is forming (148) and is found in the egg yolks (52). In the incubated hen egg, protoporphin not only develops in the embryo but increases in the egg white (by excretion from the embryo), so that approximately 130  $\gamma$  is present in the egg white of a nearly fully developed embryo (144).

Protoporphin is found in especially high concentrations in the Harderian glands of the genus *Mus* (22); these glands are claimed to be the immediate source of the porphyrin (32). A histological study by Grafflin (53) shows that porphyrin is present in the cytoplasm of the glandular cells since they exhibit a fairly intense fluorescence. Most of the porphyrin is present, however, as an accumulation of amorphous pigment granules in the excretory ducts and lamina of the glandular end pieces. The variation of protoporphin content of the Harderian gland as related to the nutrition of the rat and mouse has been studied. The pigment is said to disappear in vitamin A and B<sub>1</sub> deficiencies (53), and to decrease to about half, on feeding fats in contrast to feeding starches or proteins (137). In synthetic diets lacking pantothenic acid and choline, a "rusting" or "bronzing" appears in rats in which it is claimed that protoporphin is excreted not only from the Harderian glands, but also from sebaceous glands over the entire skin, this being associated with evident histological changes (57, 104).

Protoporphin has also been detected in barley roots, seeds, coconut milk, tree leaves, etc. (45).

**Coproporphins I and III and Uroporphin I.** These are the other naturally occurring porphins. They exist in traces and the evidence suggests that they are by-products of protoporphin synthesis (25). Two isomeric types of porphins have been observed in animals: type I represented by copro- and uroporphin, where the propionic acid groups are in positions 6 and 8; and type III, represented by coproporphin III and protoporphin IX, in which the two propionic acid groups are in positions 6 and 7. These two types cannot be derived from each other without dismembering the rings.

Coproporphin I is present in young chick embryos in less than 1% of the protoporphin which has been utilized for hemoglobin synthesis (115). It is present in amniotic fluid, meconium, and fetal serum in the last months of pregnancy (not in mother's serum), but soon disappears after birth and is then found only in traces in normal urine and stool (33,108). For example, Grotelpass isolated 96 mg. of type I and 87 mg. of type III coproporphin from 10,000 l. of normal urine. Of interest is the discovery of Klüber (80) that in the development of the central nervous system of vertebrates there is a progressive upward deposition of coproporphin in the white matter, beginning in the spinal cord and extending into the brain. Certain cranial nerves like the optic nerve were found to contain more of the porphin, and others, like the auditory, lacked it. Much has been written of the photosensitizing activity of the porphins in the presence of molecular oxygen to bring about photooxidation (137). Considering the thin skull bones of birds, it is interesting to speculate as to whether light penetrating the skull and acting on the coproporphin could induce some kind of photoperiodic effect.

In clinical cases coproporphin I is found to be excreted in increased amounts in conditions of rapid blood regeneration. This fact has been suggested to favor the hypothesis that coproporphin I is a by-product of normal protoporphin synthesis. Coproporphin III excretion is said to predominate over coproporphin I in toxic conditions such as lead poisoning and in aplastic anemias.

In the plant kingdom coproporphin has been reported present in grains in relatively large amounts (70) and in yeast (77). The yield of coproporphin I is increased if the yeast is poisoned with copper (34). Coproporphin fluorescence is strongest in dead yeast cells, in cell nuclei, and in ascospores (146).

Excretion of uroporphin I in increased amounts and its deposition in bones and other tissues is characteristic of congenital porphyria. Turner (141) has discovered that the American fox squirrel, *Sciurus niger*, is a congenital porphyrinuric since it excretes large quantities of uroporphin I. This animal should prove useful in a study of abnormal porphin synthesis. No evidence for a uroporphin type III has been found in nature (156).

Fluorescent pigments, probably coproporphins, have been noted under certain cultural conditions in a number of bacteria, especially in mycobacteria and corynebacteria. Coulter and Stone (21) observed the curious relation that increased porphin excretion in diphtheria paralleled increased toxin production. According to Pappenheimer (personal communication) the concentration of both compounds is influenced in the same way by varying the iron content of the medium.

#### D. METALLOPORPHINS OTHER THAN THOSE CONTAINING IRON OR MAGNESIUM

Of the porphins containing metals other than iron and magnesium, only copper and zinc porphins have been reported in cells, and our information about these is very

meager. The copper and zinc complexes of coproporphin and uroporphin, but not of protoporphin, have been found. The relative ease with which copper can be inserted into porphyrin at body pH makes it appear strange that the copper complex is not found more often. Indeed, when it is found, as in yeast, it is usually considered an artifact due to contamination with metal containers, etc. Copper porphyrin has been claimed by King to catalyze ascorbic acid oxidation but no effect was noted on cysteine oxidation (113).

A copper uroporphin I is contained in the flight feathers of the African genus *Turacus*. Some of the pigment excreted into the medium by diphtheria bacilli is believed to be a zinc coproporphin with possible traces of copper coproporphin (21). A copper coproporphin is probably produced in brewers' yeast.

In normal urine, traces of zinc and copper coproporphins have been claimed to be present. In contrast to the congenital variety of porphyria, there is an intermittent acute type, in which Watson *et al.* (156) and others (96,142) have shown that nearly all the coproporphin I and III is excreted as the zinc complexes.

Vanadium complexes of porphyrins derived from chlorophyll have been found in oil-bearing shales of the Silurian and later geological periods but these complexes are probably of secondary origin (140). The fact that these compounds can be isolated indicates the relatively mild conditions under which petroleum is formed.

An interesting activity of manganese protoporphin has been reported by Gjessing and Sumner (51). When combined with apoperoxidase this compound appears to have about 20-30% of the activity obtainable with iron protoporphin. The cobalt porphyrin does not possess this property. Neither manganese nor cobalt porphyrins have been detected in cells.

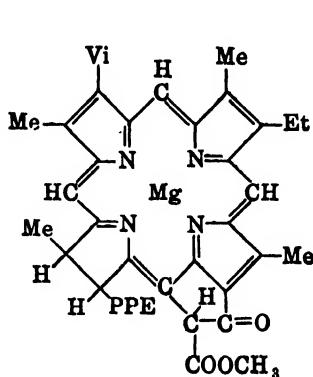
## E. MAGNESIUM TETRAPYRROLES

The porphyrins are 16-membered-ring compounds containing four pyrrole rings bound together at their  $\alpha$ -positions by  $\equiv\text{CH}$ , methene, bridges. Protochlorophyll, a pigment which is said to be precursor of chlorophyll a, is a magnesium porphyrin (39,41). Chlorophyll, a blue-green pigment, differs from protochlorophyll in the two hydrogen atoms added to pyrrole ring IV, and is therefore a dihydroporphyrin or chlorin. Bacteriochlorophyll has a tetrahydroporphyrin (bacteriochlorin) structure in which ring II as well as ring IV is reduced; here the main absorption is in the infrared. The reduction of the pyrrole rings alters the resonance and profoundly changes the character of the absorption spectra (see page 345).

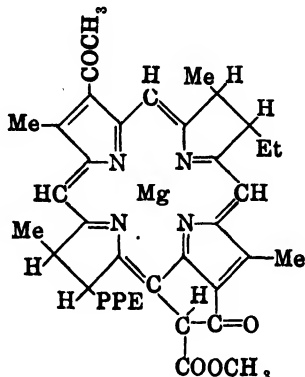
The chlorophylls are magnesium complexes containing a modified porphyrin structure isomeric with protoporphin IX. To show this relationship more clearly one may derive chlorophyll a from protoporphin IX (see page 321) as follows: The number and position of methyl side chains (Me) are left undisturbed; the vinyl group (Vi) in position 4 is reduced to an ethyl group (Et); the propionic acid group at position 6 is tied into a 5-membered ring and oxidized forming a cyclopentanone ring. The two acidic groups are esterified, one with the alcohol phytol (PPE), and the other with methyl alcohol. Two extra hydrogen atoms are added to pyrrole ring IV, and finally magnesium is inserted forming the completed chlorophyll.

Chlorophyll a is contained in all photosynthesizing plant cells. In addition, other chlorophylls occur depending on the plant. In higher plants and in green algae, the *Chlorophyceae*, chlorophyll b as well as chlorophyll a is present. It differs from chloro-

phyll a in having a formyl group rather than a methyl group in the 3 position. Chlorophylls a and c are present in the brown algae (92), and chlorophylls a and d in the red algae (128). The exact structures of chlorophylls c and d are not yet known. Bacterio-



Chlorophyll a



Bacteriochlorophyll

chlorophyll in the purple sulfur bacteria differs from chlorophyll a only in that the vinyl group is replaced by an acetyl group (Ac) and that ring II is reduced to a dihydropyrrole.

#### F. OPEN-CHAIN TETRAPYRROLES

In the red and blue-green algae the pigments phycoerythrin and phycobilin are found. These are tetrapyrrole compounds which are attached to proteins. On treatment with alkali they give rise to the open-chain tetrapyrrole glaucobilin IX $\alpha$  (119), and thus appear to be related to protoporphin rather than to the chlorophylls, as had previously been assumed. Undoubtedly, open-chain tetrapyrroles derived from chlorophyll decomposition do occur in plants but unlike those in the vertebrates, they are rapidly decomposed and their nitrogen re-utilized.

Two common open-chain tetrapyrroles of the vertebrates are the bile pigments biliverdin and bilirubin. When iron protoporphin is split at the  $\alpha$ -methene link a bile pigment compound is formed and iron is lost from the molecule. The tetrapyrrole may change its state of oxidation but its side chains remain intact for some time. Bilirubin and biliverdin are excreted into the intestinal tract where the bacteria reduce them further to mesobilirubinogen (urobilinogen) and to stercobilinogen (see pages 333 and 360).

Bile pigments have been reported from various phyla of the animal kingdom. Biliverdin has been found in egg shells of emu (138); it is the cause of the blue color of the blue coral; and it is present in the skin of a locust and in the bones of some marine fish. A blue chromoprotein isolated from cabbage butterfly wings (157) appears to contain a biliverdin isomer; this isomer may be pictured as being derived from the splitting of protoporphin IX at the  $\gamma$ -carbon position rather than at the usual  $\alpha$ -carbon position.

## G. TRANSPORT FORMS

In serum, a protein is present in the albumin fraction which, possibly because of the presence of two basic groups near each other on its surface, has the property of attaching porphins or open-chain tetrapyrroles containing propionic acid groups. This property makes it possible for the protein to transport in the blood stream compounds like ferric protoporphin, *i.e.*, Fairley's metheme albumin (31,75), coproporphin, and bilirubin.

## III. Functions of the Porphin Side Chains

Porphin by itself has no catalytic function except when iron is inserted into the ring. The specific side chains at the  $\beta, \beta'$  positions of the pyrrole rings undoubtedly are also related to the functioning of this pigment. Two approaches have been used to determine their function. One has been to vary the side chains and note how this influences the function of the modified heme protein. Another approach has been to supply modified porphins to organisms which require iron protoporphin for a growth factor and note whether these modified porphins could replace iron protoporphin in the catalytic functions essential for growth.

## A. CHANGES OF SIDE CHAINS AS AFFECTING ACTIVITY OF HEME PROTEINS

The effect of modifying the side chains of heme on the oxygen-carrying ability of hemoglobin was first studied qualitatively by Hill and Holden (65) who showed that replacement of two vinyl groups by two ethyl groups, as in iron mesoporphin, or by two hydroxyethyl groups, as in iron hematoporphin gave hemoglobins capable of combining with oxygen. Later Warburg and Negelein (151) prepared renatured globin by the acid-acetone method of Anson and Mirsky (7), coupled it with different iron porphins, and observed quantitatively the oxygen-carrying capacities of the modified hemoglobins. They showed that replacement of two vinyl by two ethyl groups as in iron rhodoporphin, or by two acetyl groups, as in iron diacetyl deuteroporphin, did not appreciably affect the oxygen-carrying capacities, nor did the replacement of one carboxyl group for a propionic acid side chain affect the activity (Table II). Iron pheophorbide b, however, could not function as a prosthetic group, no oxygen-carrying ability being observed when this compound was added to renatured globin; it is derived from chlorophyll b and contains only one free acidic group, a cyclopentanone ring, and a reduced pyrrole ring.

The effect of side chains on the activity of peroxidase has been studied recently by Theorell, Bergström, and Åkeson (135). The normal prosthetic group, iron protoporphin IX, was replaced by other iron porphins on the

TABLE II

EFFECT OF PORPHIN SIDE CHAINS ON ACTIVITY OF HEMOGLOBIN AND PEROXIDASE AND ON GROWTH OF *Strigomonas fasciculata* AND *Hemophilus influenzae*

Compounds	Side chains*					Per cent activity		Support of growth of	
	1,3,5,8	2	4	6	7	of reconstructed hemoglobin (152)	of reconstructed peroxidase (135)	<i>S. fasciculata</i> and influence on respiration (90)	<i>H. influenzae</i> smooth strains (54)
Fe protoporphin	Me	Vi	Vi	Pr	Pr	72	100	+	+
Protoporphin								+	+
Fe mesoporphin	Me	Et	Et	Pr	Pr		53	0	+
Mesoporphin									0
Fe diacetyl deuteroporphin	Me	Ac	Ac	Pr	Pr	52			0
Fe hematoporphin	Me	Hydroxy Et	Hydroxy Et	Pr	Pr		0, +†	0	+
Hematoporphin									0
Fe deuteroporphin	Me	H	H	Pr	Pr		62	0	+
Deuteroporphin									0
Fe rhodoporphin	Me	Et	Et	COOH	Pr	71	0		
Fe pyrrporphin	Me	Et	Et	H	Pr		0	0	
Pyrrporphin									0
Fe pheophorbid b†	1,5,8-triMe; 3-formyl	Vi	Et	Cyclopentanone ring	Pr	0	0	0	
Pheophorbid b									0

\* Abbreviations are: Me = methyl, Et = ethyl, Vi = vinyl, Pr = propionic acid, Ac = acetyl.

† From Gjessing and Sumner (51).

‡ Phorbin containing reduced pyrrole ring IV.



apoprotein of peroxidase. It was found that iron mesoporphin and iron deuteroporphin in which the vinyl groups are replaced by ethyl and hydrogen, respectively (Table II), resulted in quite active peroxidases. Iron hematoporphin was reported inactive although Gjessing and Sumner (51) reported an active peroxidase produced from one of their iron hematoporphin preparations. Iron rhodoporphin was inactive for peroxidase, although, as noted above, it was active for hemoglobin function. Iron pyroporphin, in which only one propionic acid group is present, was inactive.

These experiments suggest, at least for the two heme proteins hemoglobin and peroxidase, that vinyl groups are not essential for their action. It is possible to infer also that one ionizable acidic group is insufficient and that two acidic groups are essential, a carboxyl group apparently being able to replace a propionic acid group.

#### B. CHANGES OF SIDE CHAINS AS AFFECTING GROWTH OF ORGANISMS REQUIRING HEME

The effect of varying the porphirin side chains on the growth of certain trypanosomidae was first studied by Lwoff and Lwoff (90,91). These organisms require iron protoporphin for growth. When supplied with porphirins, or iron porphirins lacking vinyl groups, no growth occurred (Table II). In these organisms, heme enzymes containing vinyl groups appear to be essential for life. Cytochrome c is not among these heme enzymes, since the usual tests for the cytochrome c-cytochrome oxidase system are negative in *Strigomonas* (50). Not all the heme enzymes in *Strigomonas* need contain vinyl groups, since it could be shown that, above a certain minimum amount of iron protoporphin required for life, an additional quantity of iron deuteroporphin would further improve growth; however if the latter were too high in concentration a competitive inhibition would occur (50).

Growth of *H. influenzae* (smooth strains) proved to be an especially useful means of studying the function of the vinyl and propionic acid groups of the porphirin (54). This organism requires heme for growth. It was found that growth occurred on a number of iron porphirins even though they lacked vinyl groups (Table II). However, if iron-free porphirins were substituted for heme, no growth occurred except in the case of the vinyl-containing porphirin, *i.e.*, protoporphin. When growth occurred on protoporphin, iron protoporphin was formed by the organism. It was suggested that the vinyl groups were essential for permitting iron to be inserted into the protoporphin ring. In contrast to the flagellates like *S. culicidarum* var. *anophelis*, which required a vinyl-containing iron porphirin for growth,

*H. influenzae* grew on non-vinyl-containing iron porphins. However, *H. influenzae* when grown on the vinyl-containing porphin possessed the ability to reduce nitrate to nitrite; and it lacked this ability when grown on any iron porphin lacking vinyl groups.

The function of the propionic acid groups was also studied in relation to the growth of *H. influenzae*. When the propionic acid groups of these porphins which supported growth (*i.e.*, protoporphin, iron protoporphin, and other iron porphins) were esterified, the methyl esters no longer supported growth. Evidently two ionizable propionic acid groups on the porphins are essential for growth. They are probably required for orienting and firmly attaching the iron porphin to two basic groups of the protein so that the weak but all-important iron co-ordination link with the protein may be stabilized.

Concerning the function of the other side chains around the porphin ring, namely, the methyl groups, no direct evidence is available. From the chemical point of view a reasonable postulate is that they function as a protection for the reactive pyrrole rings, preventing them from entering into undesirable side reactions which might occur in the cells.

### C. SIGNIFICANCE OF THE STRUCTURE OF PROTOPORPHIN IX

From the foregoing survey of iron porphin proteins (Table I, page 314), it will be seen that the porphin most ubiquitous in plant and animal cells is protoporphin. This porphin is characterized by the possession of three different kinds of side chains in the  $\beta, \beta'$  positions of the pyrrole rings: two vinyl groups, two propionic acid (Pr) groups, and four methyl groups. Only minor deviations from this fundamental pattern occur in nature. All naturally occurring iron porphins possess the two propionic acid side chains. Likewise the methyl side chains are a constant feature. The vinyl side chains show less constancy. For example, in spirographin, one vinyl group is replaced by a formyl group; and this iron porphin is suggested by Keilin and Hartree (72) to be the prosthetic group of cytochromes  $a$  and  $a_2$ . Another variation of the vinyl group is found in cytochrome  $c$  where two cysteine residues of the cytochrome apoprotein may be considered to have combined with the vinyl groups to form two thio ether bonds.

If one may generalize from these data, it is suggested that the protoporphin structure is ubiquitous because it contains vinyl groups which in some way make possible the biological insertion of iron into the porphin ring. In addition protoporphin contains two propionic acid groups which appear to be necessary for orienting and firmly attaching the heme

to the protein in order to stabilize the iron linkage to another group in the protein.

#### D. COMPETITION OF PORPHINS FOR APOPROTEINS

It has been noted above that porphins lacking either the iron or the vinyl groups do not support the growth of smooth strains of *Hemophilus influenzae*. Indeed, they actually inhibit the growth which is supported by iron porphins. A competitive inhibition of growth is readily recognized between the iron porphin (for example, iron protoporphin) and the porphin into which iron cannot be inserted (for example, deuteroporphin).

In a similar manner, a competitive inhibition can be observed between two iron porphins (for example, iron mesoporphin and iron protoporphin), both of which support growth of *H. influenzae* and only one of which (iron protoporphin) effects nitrate reduction. If these two iron porphins are added together in suboptimal amounts, growth is increased over that attained by one of them alone, but the nitrite produced is less than expected from the presence of iron protoporphin alone.

For these competitions to be present one requirement is essential: the propionic acid groups must be ionizable. For example, when deuteroporphin is methylated at the propionic acid groups no inhibition of growth with this compound is observed. This is readily understandable if we assume that the propionic acid groups are required for attachment to the apoenzyme.

As we have seen, the heme proteins are the catalysts which make oxygen available to the cells. Let us assume that the chemistry of *Hemophilus* with respect to the porphins is the prototype for most cells, and let us then suppose that a porphin were produced by the cells which contained no vinyl groups. Such a porphin could not be converted by the cell to an iron porphin, although it could compete with iron protoporphin for the apoprotein enzymes which would normally attach heme at the time the apoproteins were being synthesized. In a sense, this porphin would be a natural inhibitor and would govern the relative degree of oxidation or anaerobiosis in the cell. A porphin which is widespread in nature in minute amounts, as compared to protoporphin, is coproporphin I. According to this hypothesis, coproporphin might function in this limited sense as a natural inhibitor for aerobic cell respiration.

In connection with this problem of competition, an interesting situation becomes apparent when one considers the chloroplasts. These bodies are autonomous units of remarkable synthesizing ability. They contain

chlorophyll, and there is evidence that they also contain some heme enzymes (106). In contrast to the number of heme molecules there is a relatively large number of chlorophyll molecules in the chloroplasts. If the chlorophyll molecules had ionizable propionic acid groups, they would be able to compete with heme for the specific heme apoproteins; and under such conditions no heme enzyme would become functional. However, chlorophyll differs from the porphins in such respects as to prevent competition from taking place. Thus one of the propionic acid groups is rendered inactive for protein attachment by esterification with the  $C_{20}$  alcohol phytol. The other propionic acid group is cyclized, and the resulting free carboxyl group is methylated. According to this interpretation it would seem that the plant has taken excessive measures to rid itself of ionizable propionic acid groups in chlorophyll and it may not be too hypothetical to consider that this is necessary, at least in part, to prevent competition for heme apoprotein surfaces in the chloroplasts.

#### E. PORPHIN SIDE CHAINS AND STRUCTURE OF HEMOGLOBIN

It may be well at this point to summarize the data presented thus far by using as illustration the most thoroughly studied heme protein, hemoglobin. Hemoglobin from horse red cells is a protein molecule of molecular weight 68,000, of dimensions  $64 \times 36 \times 48 \text{ \AA}$ , with four heme groups on its surface lying parallel to each other (16). In Figure 1, two heme groups are represented on the upper surface and two are implied for the under surface, although from the data one cannot exclude the possibility that all four heme groups are on the same globin plane.

Protoporphin, because of its resonating structure, is a planar molecule, of dimensions  $14 \times 17 \text{ \AA}$ . The iron in iron protoporphin is co-ordinately bound by the four nitrogen atoms of the ring with the possibility of binding one atom above and another below, the plane of the ring.

One interpretation of the structure of the globin molecule is that it is composed of four parallel monolayers of polypeptides, each  $9 \text{ \AA}$  thick. From the end group analyses of Chibnall it is suggested that each layer contains four polypeptide chains, each chain being long enough to be folded in half in the long dimension of the globin molecule (16).

When the globin molecule is newly formed in the immature red cell the heme may be imagined to diffuse into the neighborhood of the globin molecule. The two ionized propionic acid groups of the heme become attracted, oriented, and attached to two strongly basic groups on the globin surface by coulomb forces. The heme then folds onto the globin surface

where the iron of the heme comes in contact with another group, perhaps an imidazole nitrogen to which it attaches, forming the completed hemoglobin complex. This latter attachment, representing the fifth co-ordinating link of the iron is in part responsible for maintaining the iron in the ferrous state.

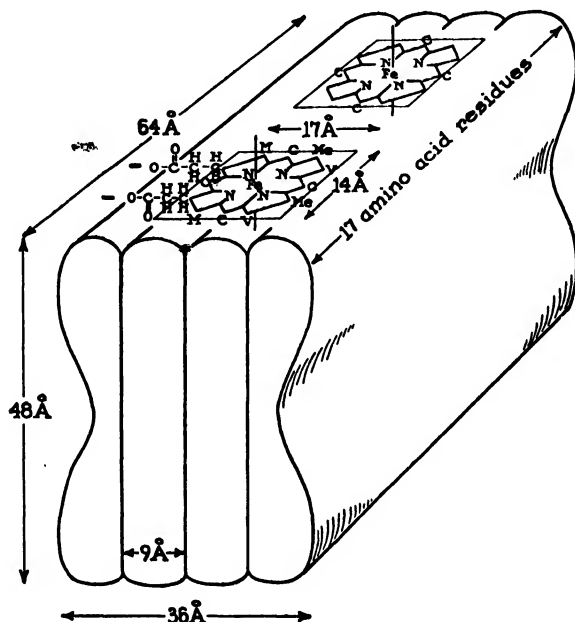


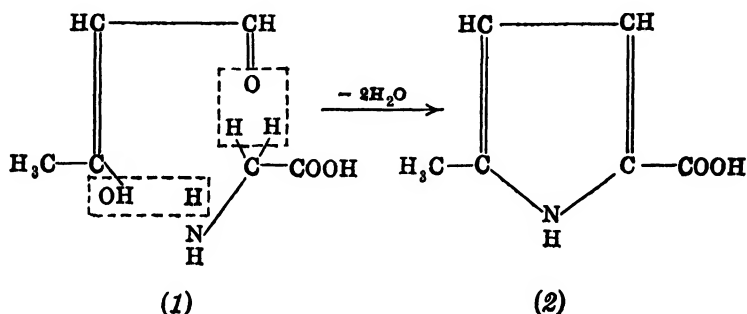
Fig. 1. Molecule of horse hemoglobin. M and Me represent methyl groups; V represents vinyl groups.

It endows the heme with the peculiar property of permitting the sixth co-ordination link to be reversibly held by an oxygen molecule. Magnetic measurements show that the unpaired electrons of oxygen, and of the iron, have all paired, resulting in a diamagnetic oxyhemoglobin, and it is perhaps this change which is significant in preventing the ferrous hemoglobin from being autoxidized.

#### IV. Porphin Synthesis

Porphin synthesis occurs at a rapid rate in two places, namely, in the immature red blood cell and in the etiolated plant leaf cell exposed to light. The first evidence of hemoglobin formation is observed in the polychroma-

tophile erythroblasts of the bone marrow; no granules or mitochondria have been identified with this process. At this early stage the cytoplasm is still predominantly basophilic due to its high content of ribonucleic acid, and the acidophilic staining is indicative of globin formation. By the time the normoblast has matured, the cell is saturated with hemoglobin, the last vestiges of ribonucleic acid being visible as the reticulum of the reticulocytes (30). In contrast to the heme in the red cell, which appears diffusely, chlorophyll is deposited in definite structures of the cell, the chloroplasts. The chloroplasts begin as tiny colorless granules which contain



the carotenoids. On exposure to light, the chloroplasts enlarge rapidly and turn green. The mature chloroplasts contain tiny ( $0.5 \mu$ ) units, the grana, in which the yellow and green pigments appear to be concentrated. Recent data of Frank (48) indicate that the chlorophyll pigments lie nearer the periphery than do the yellow pigments.

#### A. PYRROLE FORMATION

The initial step in porphyrin synthesis has recently been suggested by the fundamental discovery of Shemin and Rittenberg (117) that glycine, labeled with heavy nitrogen, is the nitrogenous precursor of the porphyrin ring. The nitrogen of ammonia, *dl*-leucine, *dl*-proline, or *dl*-glutamic acid is not effective for porphyrin production. Acetic acid or a closely related compound also appears to participate in this synthesis (15). Chemical support for pyrrole formation from glycine is suggested by a new reaction described by Fischer and Fink (35). Under mild conditions, namely, at room temperature and at pH 8, the condensation (1 above) of formyl acetone with glycine occurs rapidly, and a positive Ehrlich color reaction is obtained, indicating the presence of a pyrrole (2).

## B. DIPYRROLES, TRIPYRROLES, AND TETRAPYRROLES

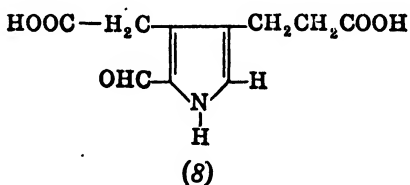
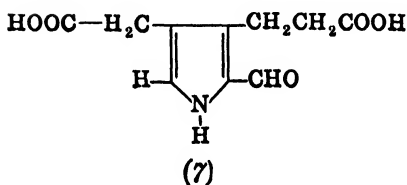
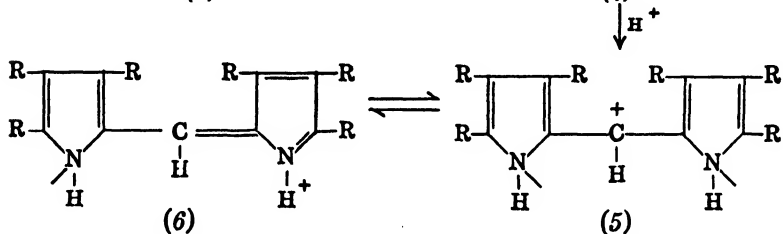
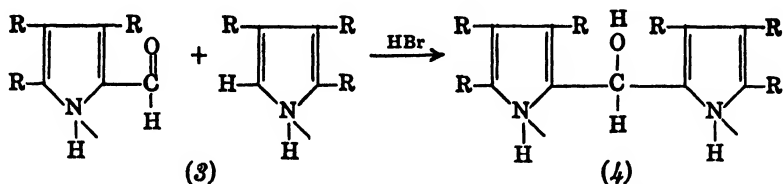
No knowledge is available concerning the normal pyrroles or dipyrromethenes which may be precursors for the tetrapyrrole protoporphin IX. The structure of coproporphin I and III and uroporphin I might be borne in mind as suggesting some clues to the main pathway of porphyrin synthesis. Also, the reactive dipyrromethene, porphobilinogen, found by Waldenström (149) and appearing in acute porphyria, might be considered in this connection. Hypotheses based on these ideas have been adequately discussed in a number of reviews (25,143,146). Although the reduced dihydropyrrole rings of chlorophyll and bacteriochlorophyll have been assumed to be derived from porphyrin rings by reduction, one should consider also the possibility that the porphyrins may be oxidation products of a dihydroporphin ring or a tetrahydroporphin ring rather than reduction products.

On paper, the simplest process that may be assumed for the biological synthesis of the porphyrin nucleus from pyrroles is a kind of aldol condensation which could occur by the addition of one pyrrole aldehyde after another, forming a *cis* configuration each time until the tetrapyrrole ring had been completed. Such a mechanism is compatible with the organic chemistry of these compounds, the unsubstituted porphyrin ring, porphyrin, having been synthesized by Fischer and Gleim (37) in small yield by heating pyrrole  $\alpha$ -aldehyde in formic acid.

Some of the intermediate steps in the synthesis of a dipyrromethene have been studied chemically (36) and the spatial changes are similar to those postulated by Michaelis (93) for the triphenylmethane dyes. The condensation of the pyrrole- $\alpha$ -aldehyde (3) goes through an intermediate carbinol stage (4) in which the central carbon atom is bound tetrahedrally. The carbinol readily loses hydroxyl ion in the presence of very weak acid to form the planar carbonium ion (5), which represents one of the resonance forms of dipyrromethene usually written as (6).

If one were to assume two isomeric pyrrole  $\alpha$ -aldehyde (7) and (8) containing acetic and propionic acid groups in the  $\beta, \beta'$ -positions, as the building blocks, it would be possible to derive all the known type I and type III porphyrins from them. By decarboxylation of the acetic group, a methyl group would arise. As pointed out by Turner (143) pyrroles of the above type are unstable and would tend to lose carbon dioxide readily (38). By carboxylation and oxidation of a propionic acid group a vinyl group might arise. Such a hypothesis would explain too much and is probably too simple.

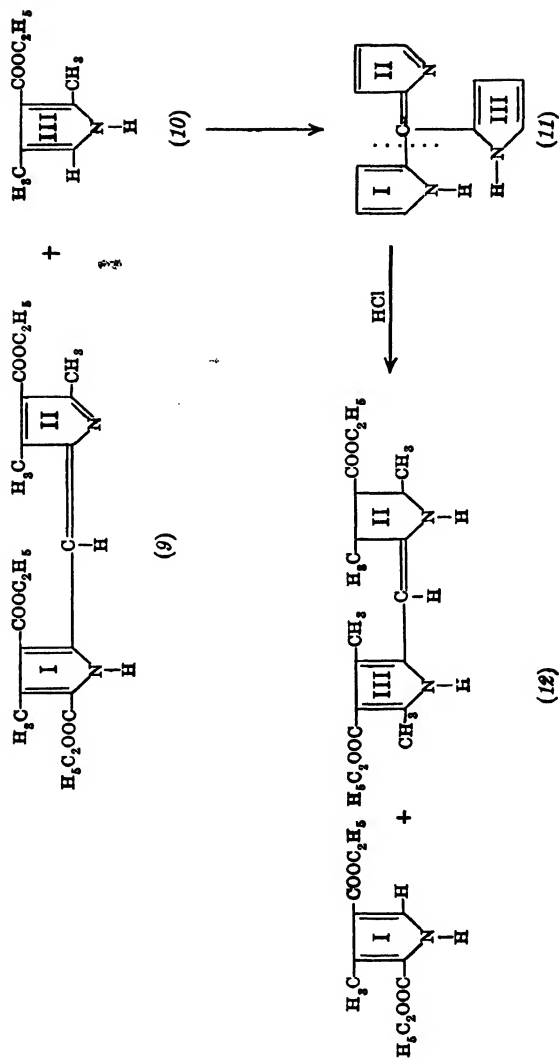
The course pursued in chemical preparations has been, for obvious reasons, the combination of two dipyrromethenes to form the porphin, and most biochemical postulates have favored such a scheme (25), although without particular justification.



A mechanism studied by Corwin and Andrews (19), which may be of biological importance, is the condensation of a dipyrromethene (9) on page 332 with a pyrrole which has a free  $\alpha$ -position (10). Here one pyrrole may exchange for another: through the intermediate formation of a tripyrrole (11), which then splits to give the new dipyrromethene (12).

One tripyrromethene has been found in nature; it is the red pigment occurring in *Serratia marcescens* (*Bacillus prodigiosus*) which, according to Wrede (159), has the structure shown at the left above.





The exchange of one dipyrrole (I) for another (III) in dipyrrolylmethene through the intermediary formation and cleavage of a tripyrrole (see page 331)

## C. FORMATION OF HEME

Further steps in the synthesis of heme have been indicated by growth studies with organisms which require heme (54,91). Evidence that protoporphin is a direct precursor of heme is shown by the fact that protoporphin when supplied to *Hemophilus influenzae*, for example, can be converted to iron protoporphin; and is supported also by the occurrence of protoporphin in immature erythrocytes, which are rapidly synthesizing heme. In the case of the "smooth" *H. influenzae* organisms, it could be demonstrated that iron is inserted into a porphin containing vinyl groups, *i.e.*, protoporphin, but not into closely related porphins, indicating that the vinyl groups play some role in facilitating the insertion of iron into the porphin ring (54). It may be surmised that the site of iron insertion into protoporphin is not necessarily at the apoprotein-heme enzyme surface since the completed iron protoporphin compound may be supplied as such in the medium and utilized by these organisms.

## V. Decomposition of Porphins and Iron Porphins

## A. DECOMPOSITION OF IRON PROTOPORPHIN TO BILE PIGMENTS

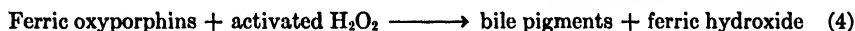
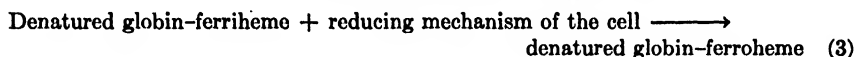
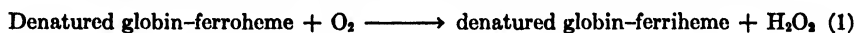
This process has been studied intensively, especially in connection with the breakdown of the hemoglobin of red blood cells to bile pigments, and our knowledge of the chemical steps taking place has been due primarily to Fischer and Libowitzky (40), Siedel (118,119), Watson (154,155), and Lemberg.

The destruction of the erythrocyte, or its fragments, and of the hemoglobin molecule itself appears to be completed in the phagocytic cells of the reticulo-endothelial system (107,111). Following phagocytosis there take place the digestion of the globin, the release of iron from the heme, and the conversion of the ring porphin to the open chain tetrapyrrole bilirubin.

The process for this conversion is an oxidative one, the heme catalyzing its own destruction, and may be pictured as follows (compare page 334). When hemoglobin is denatured, the attachment of the iron at the stabilizing link in the protein is broken, and the ferrous iron of the heme immediately gives up an electron to the oxygen molecule, resulting in the formation of ferric heme and, indirectly, hydrogen peroxide (Eq. 1). Now ferric heme avidly combines with hydrogen peroxide, and behaves as a peroxidase to activate hydrogen peroxide. This activated oxidant then either reacts with a globin molecule to begin its denaturation or oxidizes another heme molecule at a methene link (Eq. 2).

Thus a cycle is begun in which the ferric heme is reduced by cell metabolites (Eq. 3) and the ferrous heme is again autoxidized by oxygen, a ferric heme-hydrogen peroxide peroxidase compound being formed which oxidatively destroys more heme molecules. According to this scheme catalase in the red blood cells would not offer too much protection since the avidity of ferric heme for hydrogen peroxide is very great, and the hydrogen peroxide may not even come off the heme during its reduction from oxygen to hydrogen peroxide (*i.e.*, an electron donor may give up an electron to the  $\text{HO}_2$  radical-ferriheme complex). The ferric oxyporphins are then further peroxidatively oxidized to bile pigments (Eq. 4).

#### SCHEME FOR CATALYTIC OXIDATION OF HEME IN INJURED RED CELLS



The heme molecule, once its normal iron linkage to globin is disrupted, thus has the property of catalytically destroying more hemoglobin. The importance of the peroxidative property of heme in this process becomes evident when one compares the action of 0.5% hydrogen peroxide on ferriheme and on protoporphin at pH 10. Under the same conditions, heme is destroyed within a few minutes whereas protoporphin is unaffected. If, however, a mixture of heme and protoporphin is treated with hydrogen peroxide as above, both are rapidly destroyed (50).

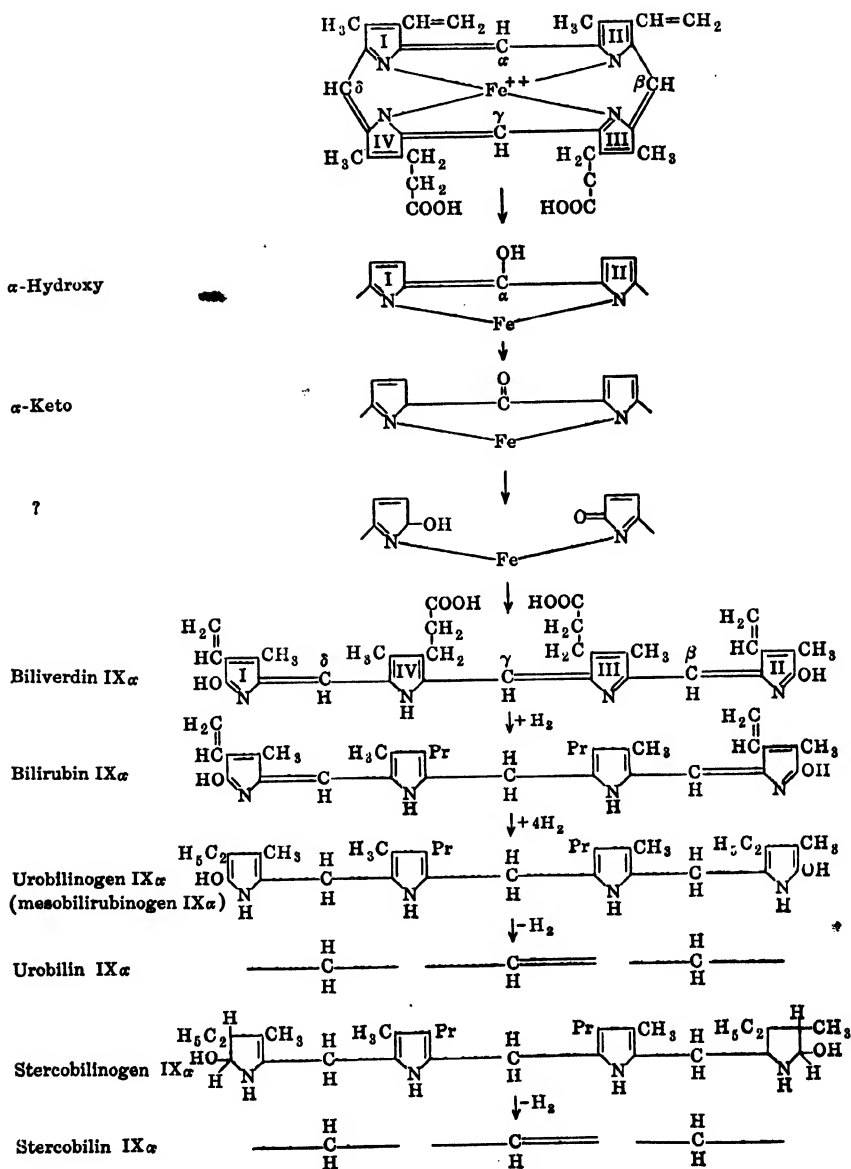
In the body, the oxidation of the heme, derived from hemoglobin takes place at the  $\alpha$ -methene link, this specific oxidation being brought about by some steric or enzymic factors as yet unknown. This may be inferred from the fact that the heme destroyed can be almost quantitatively accounted for in the bilirubin excreted. No chemical reason is evident for this biological attack at the  $\alpha$ -methene carbon atom.

In order to study the oxidative steps more readily, Libowitzky and Fischer (87,88) used a symmetrically constituted iron porphin, iron coproporphin I tetramethyl ester, in which all the C methene atoms were identical so that a better chance was given for the isolation of oxidation products. By treating this compound with hydrogen peroxide in pyridine, a green ferric compound with a band at  $655 \text{ m}\mu$  was isolated in which the methene

was oxidized to a hydroxymethene group. On shaking with air in presence of pyridine, the methene link was further oxidized to the keto group, also green with similar absorption bands. This keto compound was called a "verdo" heme to distinguish it from the green hydroxy derivative. It could be proved that the ring was intact at this stage by reducing the ferric copro-I-keto ester to coproporphin tetramethyl ester. On adding a trace of alkali to the ferric copro-I-keto ester, a yellow-brown pigment was formed which still did not give a positive Gmelin test for bile pigments. Making the brown compound weakly acid resulted in the formation of the blue-green open chain coproglaukobilin I ester and the splitting out of the iron. Although a carbon atom should have come off at this stage it was not identifiable. To complete the chemical picture, the coproglaukobilin ester which corresponded in oxidation level to biliverdin, was reduced by zinc dust and acetic acid to the corresponding bilirubin, *i.e.*, coprobilirubin I ester.

In a chemical study of the steps in the oxidation of iron protoporphin IX, complicated mixtures arose which made the interpretation of the results difficult. According to Lemberg *et al.* (86), the treatment of iron protoporphin with pyridine and ascorbic acid (hydrogen peroxide being produced as a result of the autoxidation of ascorbic acid) resulted in a green solution with absorption bands at 655, 531, and 500  $m\mu$ . A Soret band characteristic of porphins was absent, and only a low absorption between 300–400  $m\mu$  was observed (68). On the addition of acid, biliverdin and biliviolin could be isolated. The biliverdin IX dimethyl ester of correct melting point was isolated by Lemberg (84) in about 2% yield. According to Lemberg, the green compound still contained iron but he believed the ring was open and that a carbon atom had been lost. By addition of ammonia this green iron compound was readily converted in 20% yield (85) to a crystallizable iron azoporphin, in which a nitrogen united the pyrrole rings I and II, possibly lending support to the open ring structure for the green pigment.

The various data on the conversion of globin heme to bile pigments have been summarized in the scheme on page 336. As has been mentioned, the process of decomposition of iron protoporphin IX appears to be an oxidative one. When released from its normal linkage to globin (*i.e.*, on denaturation) heme catalyzes the destruction of other hemes. The oxidation is directed at the  $\alpha$ -methene carbon atom, the first oxidative product being a green  $\alpha$ -hydroxy compound, and the next one a green  $\alpha$ -keto compound. A further oxidation product for which evidence is in-



Conversion of globin-heme to bile pigments

direct and still unsatisfactory is one in which the  $\alpha$ -carbon atom has been lost, the ring has been opened, and the iron is still present. The next step appears to be a rearrangement in which the iron is lost and an open chain, blue-green tetrapyrrole compound is formed. This compound is a biliverdin IX ("IX" denotes its origin from protoporphin IX and the order and kind of side chains; " $\alpha$ " denotes that the splitting of the ring has occurred at the  $\alpha$ -methene position). Biliverdin is reduced to the orange-yellow bilidien, bilirubin, by the addition of two hydrogen atoms at the double bond attached to the central,  $\gamma$ -methene group. Bilirubin is excreted from the gall bladder into the duodenum where it is further reduced by bacterial flora. One product of intestinal reduction is the colorless, saturated bilane, mesobilinogen (urobilinogen), in which four hydrogen atoms are attached to bilirubin at the two double bonds of the chain and in which both vinyl groups are reduced to ethyl groups. This compound, in contact with the air, is oxidized at the  $\gamma$ -methene carbon atom to the orange-red bilidien, mesobilin (urobilin) IX $\alpha$ . Another colorless reduction product is stercobilinogen IX $\alpha$ . Here, not only have the double bonds along the chain been reduced, but the rings I and II at the ends of the chain have also been partially reduced, resulting in the formation of an optically active compound. In contact with air this leuco compound is oxidized at the  $\gamma$ -methylene carbon atom to a bilene, stercobilin (also orange-red).

## B. DESTRUCTION OF PORPHINS AND BILE PIGMENTS

Little is known of the destruction of the porphins themselves. Hematoporphin when injected is excreted in the bile. Coproporphin, because of its relatively greater solubility, is excreted in the urine and also in the bile (146). Injection of 100 mg. of protoporphin into bile fistula dogs resulted in the excretion of only 1 mg. (155a), possibly indicating destruction of the porphin. Kench (76) has recently found that, when coproporphin I is incubated with yeast under conditions in which coproporphin is known to be synthesized, it disappears, suggesting that the amount of coproporphin isolated from yeast at any one time is determined not only by the rate of production, but also by the rate of destruction of the porphin.

The reduction of biliverdin to bilirubin was shown to take place in the presence of liver brei (13). Baumgartel (14) claims that reduction by liver brei, especially in the presence of cysteine, secondary phosphate, and glucose, can be carried so far that even the pyrrole reaction is no longer positive. It is interesting to note that the reduction of biliverdin to bilirubin

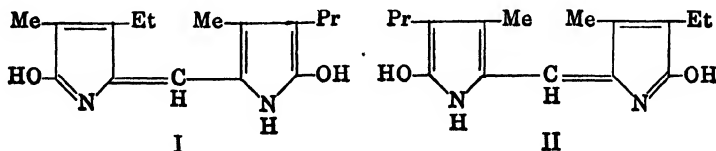
is dependent upon a specific enzyme. For example, *Escherichia coli*, in spite of its reductive capacity, is not able to convert biliverdin to bilirubin because it cannot reduce the central  $\gamma$ -methane link (14). Within a narrow pH range (around 7.4), bilirubin can be oxidized to biliverdin by hydrogen peroxide and peroxidase (129a).

In contrast to the animal which squanders its nitrogen and excretes heme in the form of bile pigment, the plant carefully conserves its nitrogen, breaking down the chlorophyll to fragments which can be transported from the older or dying leaves to storage organs or young tissues. Nothing is yet known of these intermediate decomposition products of chlorophyll.

### C. DIPYRROLE COMPOUNDS

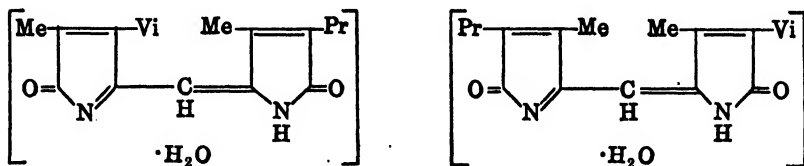
Dipyrrole compounds have been observed to be excreted in some pathological conditions, although under normal conditions it is the tetrapyrrole bile pigments which are the usual end products. In acute porphyria, Waldenström and Vahlquist (150) found a colorless compound, "porphobilinogen," in urine (101a). This compound was insoluble in organic solvents, gave a positive Ehrlich test for pyrrole, and autoxidized to a "porphobilin" which had a broad absorption band with a maximum at 500  $\mu$ . It could not be crystallized, but its properties suggest it may be a dipyrromethene-tetracarboxylic acid. It is uncertain whether this dipyrrole should be considered a step in the synthesis of tetrapyrroles or a degradation product.

Meldolesi, Siedel, and Moller (92a) obtained dipyrroles from patients with acute muscular dystrophy, and from women during the first week of puerperium when the uterus involutes. The dipyrroles were therefore considered to be decomposition products of muscle hemoglobin. The splitting of heme gave rise to two dipyrroles, the "bilifuscins," which were found in the gall bladder. In the intestines, the vinyl groups were reduced to ethyl groups resulting in the mesobilifuscins I and II; these could be isolated from the stools. Attached to protein, the brown protein material is designated as "myobilin." The mesobilifuscins did not give the "pentdyopent" test and could not be crystallized. Their suggested structures follow:



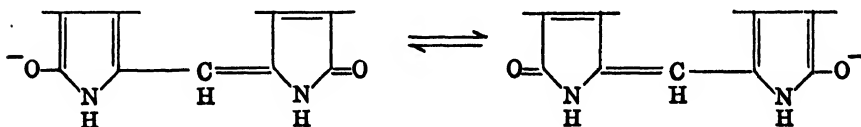
Watson (154) suggests that they may be identical with "copronigrin" and that similar products can be obtained by the oxidation of urobilin and stercobilin.

In icteric urine, colorless dipyrroles, "propentdyopents," were found (14a). The structures\* postulated for them by Fischer and Dobeneck are:



When this urine was treated with alkali and dithionite a red color was produced. The red pigments, "pentdyopents," have an absorption band in the neighborhood of 525 mμ; they are salts which autoxidize very readily to colorless forms, and they also become colorless in very weak acids. Propentdyopent was isolated from gall stones as colorless crystals by Fischer and Dobeneck (33a) and was found to consist of two dipyrroles, differing in side chain composition, and derivable from bilirubin by oxidation.

The following resonating formula is proposed for pentdyopent to account for the red color produced in alkali on reduction:



Oxidation of hemes, bile pigments, and most dipyrromethenes with hydrogen peroxide in alkaline solution gives rise to propentdyopents with different side chains. Monopyrroles, dipyrrolyl ketones, porphins, and stercobilin do not give rise to propentdyopents (42).

## VI. Physical Properties of the Tetrapyrroles

A knowledge of the physical properties of the porphins, phorbins, and bilirubinoids has played a fundamental part in the isolation and synthesis of this complex group of compounds. Studies, primarily by Stern and co-

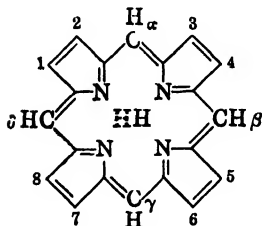
\* To account for absence of color of propentdyopent, the central C atom probably should be written here in the carbinol form.



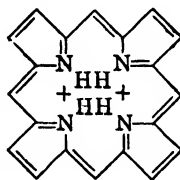
workers, using the compounds of Fischer, have already provided sufficient data to form the basis of certain rules which relate structural characteristics and absorption spectra. Other physical properties such as *pK* and fluorescence spectra have been considered in relation to chemical structure. It is hoped that these correlations will prove useful in investigations on the biochemical syntheses of the tetrapyrroles.

#### A. RING STRUCTURE OF PORPHIN

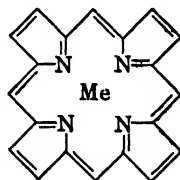
The parent substance of the ring tetrapyrroles is porphin. Because of resonance, the double bonds may be written in a number of different ways. The resonance may be considered to predominate in an inner 16-membered ring containing eight double bonds, with contributions from the resonating pyrrole nuclei. If two isomers are assumed (7a,29), the two hydrogen atoms may be written as attached in one case to two adjacent nitrogen atoms and in the other case to two opposite nitrogen atoms. If no isomers are assumed, and the evidence favors this view (10a), then one may picture the two hydrogen atoms of the neutral porphin as shared equally between the four nitrogen atoms, one hydrogen being located in the center and just below the plane of the ring (indicated as a broken H), and the other in the center and just above the plane of the ring.\*



Neutral porphin



Acid porphin



Neutral metal porphin

The uncertainty in placing the two hydrogen atoms in the porphin disappears when two protons are added in acid solution, or when the two hydrogen atoms of porphin are displaced by a bivalent metal atom. There is sufficient room in the center of the ring for all the four hydrogen atoms

\* From the spectrum of *N*-methyletioporphin II, Erdman and Corwin (30a) have recently concluded that isomers of porphins do exist. The lifetime of the H isomers may be very short, but longer than that required to absorb a photon and to lose the excited state. However, in the case of the *N*-methyl compound only one isomer was isolated.

or the metal atom to lie in the plane of the ring, and a symmetrical resonating structure is thereby attained. Conant *et al.* (18) have shown by titration in glacial acetic acid that the porphrin structure contains two weakly basic groups comparable in strength to pyrrolenine nuclei and also two very weakly basic groups comparable in strength to pyrrole nuclei. Studies of the absorption spectra of the porphins do not indicate that the two pyrrolenine nitrogens are fixed. They cannot be assigned to any particular nuclei, and one must consider therefore that the affinity for protons is a property shared by all four nitrogen atoms.

### B. RULES FOR ABSORPTION SPECTRA OF PORPHINS

The neutral porphins all have four absorption bands in the visible between 500–700  $m\mu$ , and a strong ultraviolet "Soret" band at around 400  $m\mu$ . The position of the band maxima vary depending on the solvent in

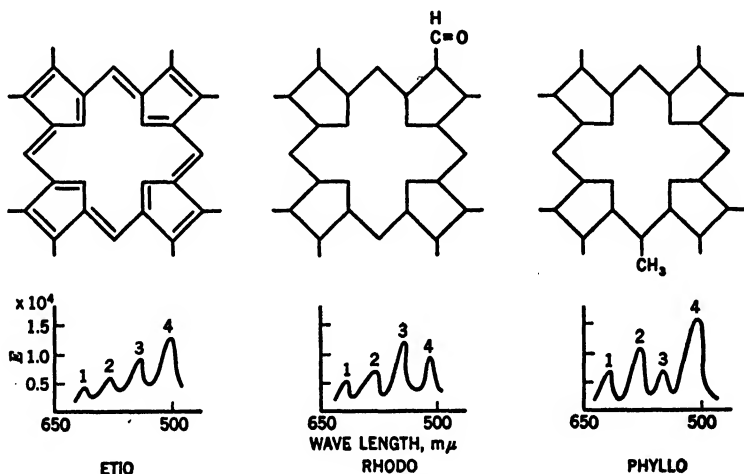


Fig. 2. Porphin-type spectra: the etio type shows the step ladder effect on the four visible bands; in the rhodo type the third band is higher; in the phyllo type the third band is lower.\*

which the porphins are measured. Most of the measurements of Stern and co-workers were made in dioxane. In general, the higher the refractive index of the solvent, the greater the band shift toward longer wave

\* *E*, in the following tables and figures, represents the molar extinction coefficient (68a).

lengths. The solvent displacement effect is less in the porphins, and greater in the dihydro- and tetrahydroporphins.

Following the classification of Stern, three types of visible spectra may be distinguished (Fig. 2). Etio type ("stepladder") has a regularly ascending series of bands; the rhodo type has a fourth band lower than the third—the third band is "too strong"; and the phyllo type has a third band that is "too weak."

(1) Porphin, the parent substance, containing no side chains, is of the phyllo type (Fig. 3, curve). As side chains are added, the third band gradually increases in height as in 1,4-dipropionic acid porphin, is almost of the etio type in 1,5-dimethyl-2,6-diethylporphin (124a) (Fig. 3, curve 2), and is of the etio type (or "stepladder" absorption spectrum) in the naturally occurring porphins (Fig. 4) in which all the pyrroles have side

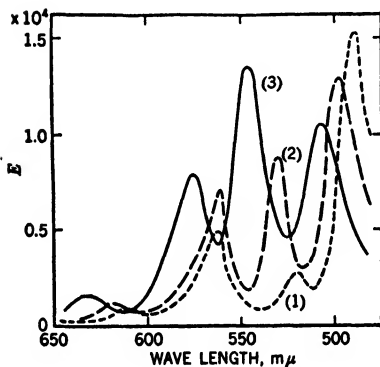


Fig. 3. Examples of porphin-type spectra in dioxane (103, 124a).

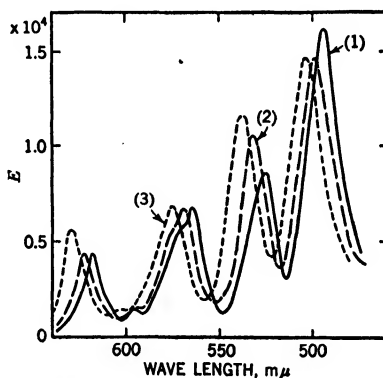


Fig. 4. Shifting of absorption bands (in dioxane) by chromophore groups (102).

chains in the  $\beta$  positions. The "stepladder" spectrum is so typical of naturally occurring porphins that it is an excellent property for their identification.

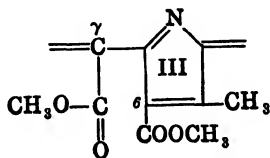
In Figure 4, curve (1) is for deuteroporphin IX dimethyl ester, (2) is for the monovinyldeuteroporphin IX dimethyl ester, and (3) for the divinyldeuteroporphin IX dimethyl ester.

(2) In the fully substituted porphins, changing the relative positions of the side chains (with the exception of the C=O groups) to form different isomers has no effect on the absorption spectrum. Substitution of one

nonchromophore group such as methyl, ethyl, acetic, propionic for another does not affect the absorption spectrum. Addition of chromophore groups (*e.g.*,  $\text{—HC=O}$ ,  $\text{—CH=CH}_2$ ,  $\text{—COOH}$ ) directly to the resonating ring either at the  $\beta$ -pyrrole position or on the methene bridge carbon atoms displaces all the bands, including the Soret band (*i.e.*, the strong ultraviolet band) to longer wave lengths. Some of the bands may be shifted a little more or a little less in this direction. The degree of displacement decreases in the order formyl, vinyl, carboxyl. For example, one vinyl shifts the spectrum about  $4\text{ m}\mu$ , and two vinyls about  $8\text{ m}\mu$  to the red (Fig. 4). The porphin containing two vinyl groups, *i.e.*, protoporphin IX, may be distinguished from the other naturally occurring porphins because of the displacement of the etio type bands to longer wave lengths. The shift caused by the chromophore groups is present in all of the tetrapyrrole spectra, including the porphins, dihydroporphins, acid forms, and metal complexes.

(3) A carbonyl group (or cyano) attached to the  $\beta$ -position of the pyrroles, in addition to shifting the bands to longer wave lengths, also changes the relative heights of the visible bands, so that band 4 is lower than band 3 (*i.e.*, the rhodo type). For example, in *Spirographis* porphin replacement of a vinyl in the 2-position by a formyl changes the etio type to the rhodo type (103) (Fig. 3, curve 3, for 1,3,5,8-tetramethyl-2-acetylporphin-6,7-dipropionic acid methyl ester). Acetyl, propionyl, benzoyl, or carbomethoxy groups in the  $\beta$ -position have a similar effect.

(4) When two carbonyl groups are attached to opposite pyrrole rings (*i.e.*, rings I and III or II and IV), the rhodo type effect is enhanced (*e.g.*, 2-acetyl, 6-carbomethoxy, 1,3,5,8-methyl, 7-propionic acid). However, when the two carbonyl groups are on adjacent rings (*e.g.*, 2,4-diacetyldeuteroetio porphin III), or, when one carbonyl is in the 6-position and

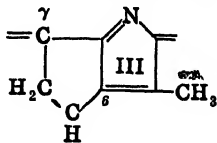


**Pheoporphin  $a_7$**

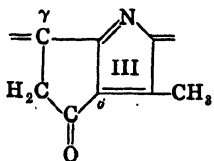
another on the  $\gamma$ -carbon (*e.g.*, pheoporphin  $a_7$  trimethyl ester), the bands take on the appearance of the etio type (Figure 2, page 341).

(5) The compounds containing a bridge of atoms between the  $\gamma$ -carbon atom and the carbon atom in 6-position have been studied especially

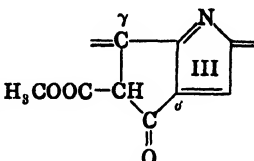
in connection with the structure of chlorophyll. The simplest bridge of this kind is a 6- $\gamma$ -ethane bridge (a)—*e.g.*, desoxophylloerythrin, which has a spectrum of the phyllo type (Fig. 2, page 341). In the ethanone derivative, phylloerythrin (b), the rhodo type is established because of the conjugation of the carbonyl group with the resonating porphin. The pheoporphin *a<sub>s</sub>* ester which has the cyclopentanone ring of chlorophyll (c) is also of the rhodo type.



(a)



(b)



(c)

(6) The addition of a hydroxyl group or an oxy group directly on a methene bridge carbon atom (*i.e.*, in the oxidation of the porphin ring) produces marked changes in the absorption spectra. Such compounds have not yet been studied quantitatively although they are of interest in connection with investigations on the splitting of the porphin ring (see pages 333-337). The hydroxy derivative of coproporphin I is reported to be blue, having a strong first band and three weak ones in the visible region (88). It fluoresces dark red and is easily decomposed by light. In 5% hydrochloric acid it fluoresces violet red and has an ultramarine blue color with two absorption bands, the first stronger band being at 619  $m\mu$ . By acetylation or benzylation, the acetoxy or benzoxy derivatives are formed which have a red color and a phyllo type spectrum so that these residues behave here as nonchromophores. The keto group at the bridge carbon might be expected to prevent resonance around the ring and its spectrum might be predicted to be related to those of the bilirubinoids. From the little data available, it seems that the absorption spectra of the hydroxyporphins may at times resemble those of the chlorophylls and of the phorbins.

(7) Addition of one bromine atom to the  $\beta$ -positions of the pyrroles does not change the character of the spectrum. When two bromine atoms are present in adjacent rings (that is, rings I and II) the etio type is found; if they are in opposite rings (that is, rings I and III) the rhodo type is found. One or two nitro groups in the  $\beta$ -pyrrole positions do not change the type spectrum, but only behave as chromophores to shift the spectrum toward the longer wave lengths (121a).

## C. RULES FOR ABSORPTION SPECTRA OF DIHYDROPORPHINS

The dihydroporphin skeleton constitutes the basic structure of the chlorophylls. Reduction of porphyrin by addition of two hydrogen atoms to pyrrole ring IV causes a striking change in the absorption spectrum as compared to the porphyrin types. The whole spectrum is shifted to the longer wave lengths. The spectrum consists of two main bands. The first band, in the red, is in the neighborhood of  $660\text{ m}\mu$  and is very high, the other visible bands being relatively insignificant. The ultraviolet band is around  $430\text{ m}\mu$  (*i.e.*, about  $30\text{ m}\mu$  to the red of the porphyrin Soret band). This characteristic group of bands is designated as the "chlorin type" (Fig. 5) (123).

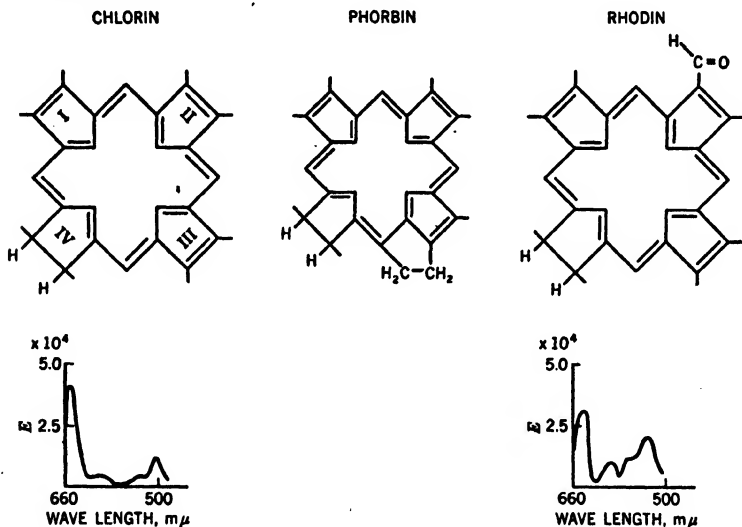


Fig. 5. Dihydroporphin rings and type spectra. The chlorin type shows one prominent band in the visible at  $660\text{ m}\mu$ ; the phorbin ring has a chlorin-type spectrum. The rhodin type has two distinct visible bands at about  $650$  and  $525\text{ m}\mu$ . See formula on page 306 for numbering of side chains and methene carbon atoms.

(1) The chromophores which are present as substituents (including carbonyl groups in rings I and III) tend to shift the absorption band to the longer wave lengths.

(2) The addition at the  $\gamma$ -methene carbon atom of a methyl, acetic acid, or carbomethoxy group does not change the spectrum appreciably. However, an aldehyde group (*e.g.*, formyl or glyoxylic acid ester) acts as a

TABLE III  
POSITION AND INTENSITY OF ABSORPTION BANDS OF PORPHINS CONTAINING THE CYCLOPENTANONE RING (60)

Porphin		Dihydroporphin									
		Chlorins						Rhodins			
Pheoporphin a <sub>8</sub> (2-ethyl)		Dihydropheophorbid a (2-ethyl)		Pheophorbid a (2-vinyl)		Chlorophyll a		Pheophorbid b (2-vinyl, 3-formyl)		Chlorophyll b	
λ, mμ	E	λ, mμ	E	λ, mμ	E	λ, mμ	E	λ, mμ	E	λ, mμ	E
420	209,000	405	145,000	412	121,000	431	143,000	434	176,000	450	209,000
520	8,910	498	10,900	503	29,200			529.5	11,000		
560	14,800					609	13,600			589	15,000
583	12,400					656	72,300	651	31,800	638	72,000 (?)
632	1,700	654	42,800	663	48,900						

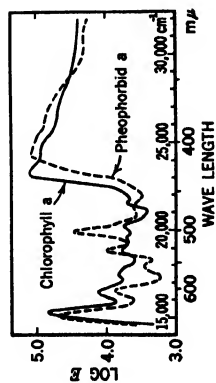


Fig. 6. Absorption spectra of pheophorbid a and chlorophyll a (60).

chromophore shifting the bands to longer wave lengths. The formation of a phorbin structure, which is characterized by a five-membered isocyclic ring between the  $\gamma$ -carbon atom and the carbon atom in 6-position (Fig. 5) does not change the character of the spectrum(122). But a keto group in the 9-position (as the ring in chlorophyll, page 321) shifts the spectrum to the red as is to be expected of a chromophore group.

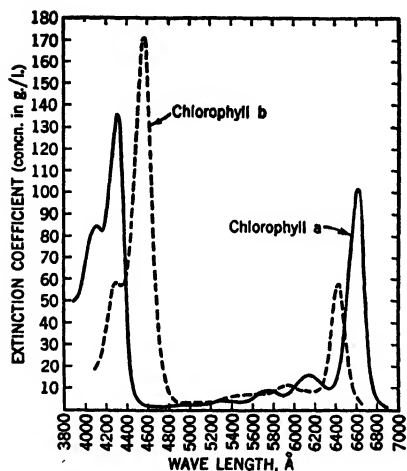


Fig. 7. Spectra of chlorophylls a and b in ether (161).

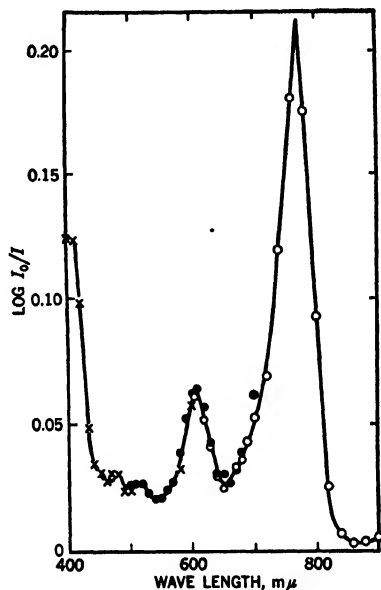


Fig. 8. Spectrum of bacteriochlorophyll in cells of *Spirillum rubrum* (49).

(3) When a formyl group is added at position 3 one obtains derivatives of the chlorophyll b series and a *rhodin-type* spectrum (Fig. 5). The C=O group in this position appears to have a marked effect on the vibration of the molecule, perhaps because it is a substituent in ring II, *i.e.*, opposite the dihydropyrrole ring IV. A C=O group in rings I or III does not change the spectral type but merely behaves as a chromophore. As compared with the chlorin type, the rhodin type (Figs. 5 and 7) has two main bands which are closer together; the first band is depressed and the ultra-violet band is heightened (Table III).



(4) Insertion of magnesium into the chlorin and rhodin does not change the type, but brings the two main bands closer together (Table III and Figure 6). Figure 6 shows bands for pheophorbide a, and for chlorophyll a which has magnesium inserted in the ring.

In the tetrahydroporphins, pyrrole rings II and III are reduced. The

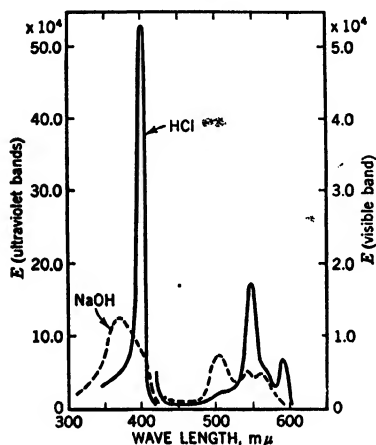


Fig. 9. Absorption spectra of coproporphins I and III (69).

magnesium complex of a tetrahydroporphin is represented in nature as bacteriochlorophyll (page 321). Its absorption spectrum is characterized by two bands in the infrared around 780 (Fig. 8) and 850  $m\mu$ .

In considering the relation between the spectra and the state of reduction, Rabinowitch (105) interprets the porphyrin absorption spectra as made up of two electronic levels, the ultraviolet Soret absorption representing the higher electronic level, and the four visible bands representing vibrational states of a lower electronic level. In the dihydroporphins a still lower electronic level is present, and in the tetrahydroporphins with the band in the infrared a new low electronic excited state is encountered.

#### D. EFFECT OF pH ON ABSORPTION AND FLUORESCENCE SPECTRA

(1) In acid solution (*e.g.*, 1 *N* hydrochloric acid) two pyrroline nuclei of porphyrin combine with two protons of approximately the same *pK* value to form a symmetrical resonance structure (page 340). Differences between neutral and acid porphyrins are reflected in their absorption and fluorescence spectra. Compared with the four-banded neutral form in the visible, the absorption spectra of the acid forms have two main bands in the visible, and a markedly higher ultraviolet band (Fig. 9). The figure shows bands for coproporphins I and III in 0.15 *N* and in 0.1 *N* NaOH (alkaline spectrum for polymerized porphyrin). The curves in the visible are ten times higher than in the ultraviolet. In phylloerythrin, a shift of the visible bands to the red is observed in 96%  $H_2SO_4$ , which is correlated with oxonium formation of the oxygen at the isocyclic ring (7a).

(2) The fluorescence spectra of porphins are a kind of mirror image of the absorption spectra. The complete fluorescence spectrum is generally excited by any wave length in the neighborhood of any one absorption band. The emitted light is red and consists of a series of bands, the most intense fluorescence band being at a wave length just toward the red of the first absorption band (*e.g.*, as in protoporphin and etioporphin, Fig. 10). Only when the tetrapyrrole chain forms a closed ring is there a strong fluorescence with band structure. Open-chain tri- and tetrapyrroles show only a diffuse fluorescence between 600 and 640  $m\mu$  and at most only a single fluorescence band.

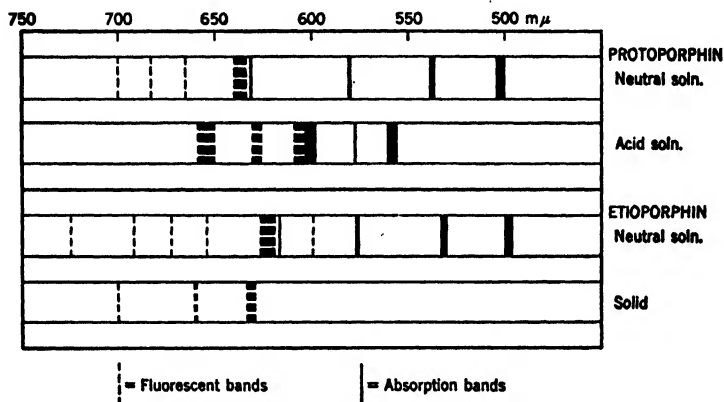


Fig. 10. Positions and relative intensities of absorption and fluorescence bands of porphins.

The neutral porphins have one main and three weak fluorescence bands whereas the acid form has three prominent fluorescence bands (Fig. 10). Studies of Dhéré (23,24) have revealed a whole series of still weaker fluorescence bands extending into the infrared. The solid porphins have absorption spectra shifted toward longer wave lengths, and they show emission spectra also displaced to the red (*e.g.*, solid etioporphin, Fig. 10).

The red fluorescence of the acid forms when excited by light of 380–430  $m\mu$  (*i.e.*, in the neighborhood of the Soret band) is one of the most sensitive means of detecting and quantitatively estimating porphins, as little as 0.1  $\gamma$  per cc. being measurable in a fluorometer. In aqueous solutions slight changes in pH or salt content, or higher hydrochloric acid concentrations, tend to decrease the absorption or fluorescence, this effect being in

part ascribable to aggregation of the porphin. A possible effect of aggregation is seen in Figure 11—the height of the ultraviolet absorption band of coproporphin in 7 *N* hydrochloric acid is displaced and is lower than in 0.15 *N* hydrochloric acid. At still lower acid concentrations the neutral form of coproporphin with its lower extinction begins to appear.

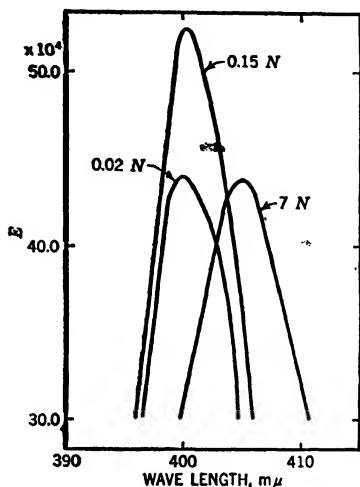


Fig. 11. Effect of hydrochloric acid concentration on position and height of ultraviolet absorption of coproporphin (69).

Curves for intermediate hydrochloric acid concentration lie between these extremes. Slightly polymerized molecules still fluoresce but with less total intensity than the monomers. The tendency to polymerize may be diminished by using organic solvents such as alcohol.

(3) In the dihydroporphins Conant (18) noted only one basic group comparable in strength to a pyrrolenine nucleus, and another still weaker base, higher acidities being required to add the second proton. No studies appear to have been made for the purpose of distinguishing between the absorption spectrum arising from the addition of the first proton and that arising from the addition of the second proton. On addition of acid the high first band is depressed and shifted to shorter wave lengths (about 20  $m\mu$  in the case of

mesorhodochlorin) but the general character of the absorption curves remains unchanged. Here, as in the porphins, the fluorescence is red, and the fluorescence bands are on the long wave length side of the first absorption band.

#### E. PHOSPHORESCENCE SPECTRA

In addition to fluorescence emission bands, some of these compounds may show phosphorescence emission bands which are on the long wave length side of the fluorescence spectra. Calvin and Dorough (16a) have shown that zinc chlorin, and a mixture of chlorophylls a plus b, possess phosphorescence bands beginning in the range of 7800 to 8000 Å and extending into the infrared. The lifetime of the phosphorescent state was about 0.2 second. Copper chlorin, which does not show fluorescence, also does not show phosphorescence.

When a molecule absorbs light, its energy may be transformed in various ways. It may be almost immediately re-emitted, within  $10^{-8}$  second, as fluorescence radiation. Or it may pass into a metastable excited state and after a prolonged time, even of several seconds, pass to the ground state and emit the energy as phosphorescence radiation. Or the electronic energy instead of causing fluorescence or phosphorescence may be degraded by collisions, etc., into heat energy. Recently Lewis and Kasha (86b) and Lewis and Calvin (86a) showed that a molecule in this prolonged excited state of phosphorescence is a biradical, *i.e.*, though the sum total of all the electrons is still even, two of the electrons no longer are paired in opposite spin. Such an excited molecule is paramagnetic and is said to be in the *triplet state* by spectroscopists. It is suggested by Calvin that the phosphorescent state of chlorophyll may be of significance for the process of photosynthesis.

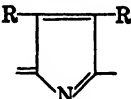
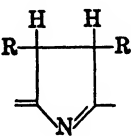
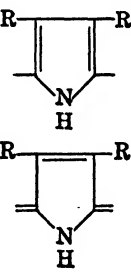
#### F. SOLUBILITIES AND $pH$

The effects of the differences in solubility due to the side chains, and the differences in  $pK$  at which the protons are added at the pyrrolenine nitrogens, may be combined to bring about the separation of closely related porphins. A method based on a combination of these properties was first systematically applied and used with great skill by Willstätter (158). The Willstätter method consists of the solution of the porphins in ether and extraction with aqueous hydrochloric acid of different concentrations. The "HCl numbers" are characteristic physical constants of the porphins. The "HCl number" is defined as that per cent of aqueous hydrochloric acid which will extract two-thirds of the porphin from water-saturated ether into an equal volume of the aqueous layer. The ether solution is made up to contain 20 milligrams per cent porphin or a saturated solution containing less.

Aliphatic side chains increase the solubility in ether and shift the HCl number to higher values. For example, the addition of a phytol group to pheophorbide a (Table IV) diminishes the solubility of the resulting compound to such an extent that it can only be extracted from ether by a strong hydrochloric acid solution which itself dissolves appreciable quantities of ether.

The relative  $pK$  values of the ring nitrogen atoms may be estimated from the HCl numbers. Or they may be determined directly by titrating the weak bases potentiometrically in glacial acetic acid with perchloric acid using the chloranil electrode (18). In a comprehensive analysis of this

problem Conant, Chow, and Dietz determined the  $pK_{\text{HAc}}$  ( $pK$  in acetic acid) values for a large number of related pyrroles. Three classes of nitrogen bases were indicated by this study. Their formulas and  $pK_{\text{HAc}}$  values are shown in the accompanying table. In dipyrromethanes, as would be

Class No.	Class Name	Formula	$pK_{\text{HAc}}$
1	Pyrrolenine <small>pyr.</small>		+2.0 to +2.2
2	Dihydro- pyrrole		-1.4 to +0.4
3	Pyrrole  Isopyrrole		-1.9 to -2.4

expected, the two very weakly basic nitrogen atoms lie in the third class. In dipyrromethenes, one nitrogen is in the third, and one in the first class. In porphins, two nitrogen atoms are in the third and the two others in the first class. In dihydroporphins (*e.g.*, chlorin) two nitrogen atoms are in the third class, one in the second, and the other in the first class.

The HCl numbers reflect the  $pK$  at which protons are added to the "strongest" of these bases (*i.e.*, the pyrrolenine nitrogen atoms). On the other hand, the titration in glacial acetic acid gives poorly defined  $pK_{\text{HAc}}$  values in this region but it is entirely satisfactory for values around  $pK_{\text{HAc}} = 0.0$  (*i.e.*, dihydropyrrole compounds). Since the substituents influence the  $pK$  values of the pyrrolenine nitrogen atoms changes due to substituents can only be inferred from the HCl numbers.

The following rules may be inferred from the data (Table IV, page 354) relating the effect of side chain substituents to the  $pK$  values of the nitrogen atoms in the tetrapyrrole rings:

(1) The attachment of different alkyl or acid groups (*e.g.*, methyl and acetic) to the  $\beta$ -positions of the pyrrole rings in porphin, or their arrangement around the ring, does not change the  $pK$  value.

(2) A chromophore (*e.g.*,  $-\text{COOH}$  and  $-\text{CH}=\text{CH}_2$ ) attached to the  $\beta$ -position makes the  $pK$  of the pyrrolenine nitrogen atoms more negative (*i.e.*, increases the HCl numbers).

(3) On the other hand, the attachment of a methyl or acetic acid group to the  $\gamma$ -carbon position makes the  $pK$  somewhat more positive.

(4) The formation of an ethane bridge between the  $\gamma$ -carbon and the 6-carbon atom does not affect the  $pK$  appreciably. However, when a  $\text{C}=\text{O}$  is present in the bridge attached to the 6-carbon atom (phylloerythrin) a strong chromophore effect is produced and the  $pK$  is more negative.

(5) In the dihydroporphins, the HCl numbers only reflect the addition of the first proton to chlorin *e*, the reduced pyrrole ring having a slight depressing effect on the  $pK$  of the pyrrolenine nitrogen. In rhodin *g*, the addition of the chromophoric formyl group depresses the  $pK$  markedly.

(6) It is only by the titration method that the weaker dihydropyrrole nitrogen can be detected. In pheophorbid *a* the chromophore in the cyclopentanone ring depresses the  $pK$  of the dihydropyrrole nitrogen in ring IV. In pheophorbid *b* the formyl and cyclopentanone chromophores depress the  $pK$  of the pyrrolenine as well as of the dihydropyrrole nitrogen atoms.

As can be seen from Table IV isomers cannot be separated by the HCl method since their HCl numbers are almost, if not identical. The separation of copro from protoporphin is readily accomplished because the HCl numbers differ appreciably from one another; indeed, it is the method of choice in analyzing tissues for naturally occurring porphins. Uroporphin I at  $pH$  4 is practically insoluble in acetic or ether and can thus be separated from the other porphins.

In the synthesis of porphins from dipyrroles this separation technic is applied systematically to isolate a number of possible products from a mixture. The ether solution containing the porphins is extracted with 0.01% ammonium hydroxide and the dicarbonic acid porphins enter the aqueous solution. The monocarbonic acid porphins are then extracted from the ether with small amounts of 10% sodium hydroxide. The third

TABLE IV. THE HCl NUMBERS, BASICITIES, AND MELTING POINTS OF SOME PORPHINS AND DIHYDROPORPHINS\*

Compound	Side chains	HCl Number†		Melting point (°C.) of methyl ester	Relative basicities in glacial HAc‡		
		Free	Methyl ester		pK <sub>1</sub> '	pK <sub>2</sub> '	pK <sub>3</sub> '
Porphins							
Copro I	(Me) <sub>4</sub>	0.08	1.5	256			
Copro III	(Me) <sub>4</sub>	0.09	1.5	130, 160**			
Uro I	(Ac) <sub>4</sub>		7	284			
Hemato IX	(Me) <sub>4</sub>	0.1		212			
Deutero IX	(Me) <sub>4</sub>	0.4	2.0	238			
Meso IX	(Me) <sub>4</sub>	0.5	2.5	216			
Proto IX	(Me) <sub>4</sub>	2.0	5.5	228			
Phyllo	(6-H)	0.35	0.9				
Phylloetio V	(6-H)	1.0	2.5				
Pyrro	(6-H)	1.5					
Deuteroetio II	(6-H)	1.8					
Pyrroetio V	(6-H)	2.5					
Rhodo	(6-COOH)	3.0					
Chloro es	(6-COOH)	1.0					
Bromodeuteroetio II	(H) (Br)	5.0					
Bromopyrro XV	(Me) <sub>4</sub>	7.5	9.0				
Dibromodeuteroetio II	(Me) <sub>4</sub>	12.0					
Desoxyphylloerythrin	(Me) <sub>4</sub>	1.8					
Phylloerythrin	(Me) <sub>4</sub>	8.0					
Phycorophin as	(Me) <sub>4</sub>		8.5				
Dihydroporphins							
Chlorin e	(Me) <sub>4</sub>	3.0					
Rhodin g	(Me) <sub>4</sub> (3-CHO)	9.0					
Phaeophorbide a	(Me) <sub>4</sub> (6-COOH)	15.0					
methyl ester	(Like chlorophyll a but minus magnesium and phytol)	16.0					
phytyl ester		29.0					
Phaeophorbide b	(Like chlorophyll b but minus magnesium and phytol)	19.5					
methyl ester		21.0					
phytyl ester		35.0					

\* Abbreviations are: Me = methyl, Et = ethyl, Pr = propionic acid, Ac = acetic acid, Vi = vinyl, Cpo = cyclopentanone ring, Cp = cyclopentane ring. † For other compounds see Kirstahler's compilation (79). \*\* Values by Jope and O'Brien (89). ‡ Grimstein (55) gives 148° and 170° for copro III ester, double melting point. † Conant, Chow, and Dietz (18).

fraction which is not extracted by alkaline solutions remains in the ether solution. These three fractions are then separated further by the aqueous hydrochloric acid-ether method (46).

Chromatography has been applied with increasing frequency in the last few years for the concentration and isolation of the porphins. When coupled with elution by means of different buffers of different pH values, separations are greatly aided (55,76,109,150). Based on such methods, the porphins of acute and chronic porphyrias have been firmly established. The isomeric porphins (as, for example, coproporphin I and coproporphin III) are characterized by the melting points of their esters. The work of Grinstein and of Watson (55,156) has demonstrated the necessity for carefully chromatographing porphin mixtures as a preliminary step, since there is a tendency for isomeric porphin esters to form mixed crystals having apparently constant melting points. Jope and O'Brien (69) have shown that the presence of 10-15% of one isomer may remain undetected in the other if the melting point is the only criterion. They have reported that the resolidification point is a more sensitive index of purity; the resolidification point is the temperature at which double refraction appears on cooling.

#### G. METALLOPORPHINS

Although a large number of metals can be incorporated into the porphin ring, only the iron and magnesium complexes occur abundantly; of the remainder only the copper and zinc complexes have been reported in traces and under special conditions.

As remarked above, the addition of two protons to the pyrrolenine nitrogen atoms of porphin produces a symmetrical resonance structure with two sharp and intense bands in the visible instead of the four bands of the neutral molecule. It is reported that a similar absorption occurs when two potassium or sodium ions are attached to the porphin in nonaqueous solvents (43,63).

When a divalent metal ion is inserted into the porphin it may be pictured as displacing two protons, the metal atom co-ordinating with the four nitrogen atoms, producing here also a symmetrical resonance structure (page 340). The absorption spectra in general consist of two strong bands, the maxima being in the range of 520-540, and 550-580 m $\mu$ . The acid porphins have bands displaced somewhat more to the red (555 and 595 m $\mu$ ). The relative intensities of the two bands vary, depending on the compound. In Table V the stronger absorbing band has been *italicized*. Although the absorption bands of the metalloporphins are all below 600



m $\mu$ , magnesium pheoporphin  $a_8$  has a stronger band at 615 m $\mu$  and a weaker one at 580 m $\mu$  (Fig. 12), indicating the strong displacement effect of the chromophore group in the cyclopentanone ring.

The metal complexes of manganese, iron, and cobalt are exceptions to the above rule in a number of ways. Of all the metalloporphyrins, only these three can be reversibly oxidized and reduced. Relatively, the iron complexes have the highest potentials, those of cobalt are lower, and those

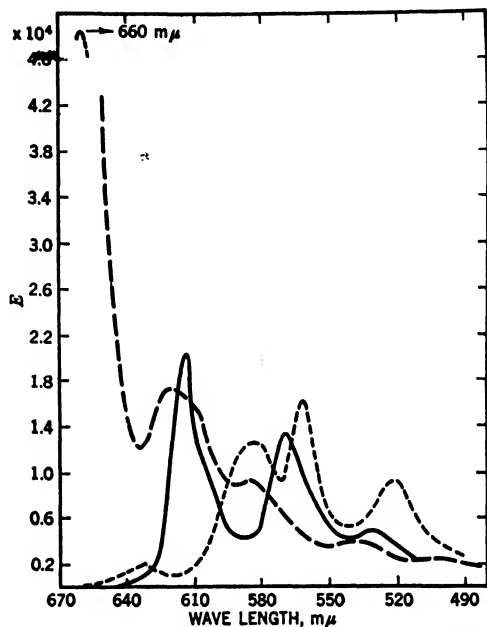


Fig. 12. Absorption spectra (124) of magnesium salt of pheoporphin  $a_8$  dimethyl ester (—); pheoporphin  $a_8$  dimethyl ester (---); and methylchlorophyllid  $a$  (— · —).

of manganese have the lowest potentials (12,130). For example the  $E'_0$  values of the iron, cobalt, and manganese mesoporphyrins in aqueous pyridine are, respectively:  $-0.063$ ,  $-0.265$ , and  $-0.387$  v. Their spectra in aqueous solution are rather broad with the exception of the intense band of the  $Mn^{3+}$  complex. Because of their tendency to form octahedral complexes with a total of six bonds, they have the important property of being able to co-ordinate with two atoms or atom groups in addition to those of the four nitrogen atoms in the porphyrin. When they co-ordinate with ni-

trogen compounds their potentials are modified. According to Taylor (130) manganous porphyrin co-ordinates with pyridine, and cobaltic porphyrin co-ordinates with cyanide. This co-ordination property is greatly enhanced in the iron porphyrins (12). Absorption spectra of these hemochromogens have been studied more recently by Drabkin (26,28); details of these spectral relationships cannot be discussed here. For ultraviolet absorption bands of some metalloporphyrins see Holden (67) and Paic (98).

TABLE V  
VISIBLE ABSORPTION AND FLUORESCENCE BANDS OF METALLOPORPHYRINS\*

Co-ordinating elements	Absorption bands, m $\mu$	Fluorescence bands, m $\mu$	Ref. No.
H <sub>2</sub> <sup>+</sup>	592 <i>555</i>	597	62
Na <sub>2</sub> <sup>+</sup>	592 555	597	43,63
K <sub>2</sub> <sup>+</sup>	595 564	599	43,63
Mg <sup>2+</sup>	581 545	586	62
Cd <sup>2+</sup>	578 543	581	62
Zn <sup>2+</sup>	575 535	582	62
Sn <sup>2+</sup>	575 540	581	62
Fe <sup>2+</sup>	<i>548</i> 519		62
Fe <sup>3+</sup>	597 490		
Co <sup>2+</sup>	545		130
Co <sup>3+</sup>	560 520		
Mn <sup>2+</sup>	585 <i>550</i>		130
Mn <sup>3+</sup>	560 <i>470</i>		62
Ag <sup>2+</sup>	<i>555</i> 520		62
Cu <sup>2+</sup>	<i>562</i> 526		120
Ni <sup>2+</sup>	<i>553</i> 517		120
Pd <sup>2+</sup>	<i>540</i> 510		120
Hg <sup>2+</sup>	565 530		120
Tl <sup>2+</sup>	580 <i>545</i>		120
V <sup>4+</sup>	580 <i>545</i>		120
Sn <sup>4+</sup>	<i>575</i> 535		120
Ga <sup>4+</sup>	<i>575</i> 535		120
In <sup>2+</sup>	<i>575</i> 535		120

\* Stronger absorption bands are italicized.

Of all the metal porphyrins that have been prepared, only the magnesium, cadmium, zinc, and tin complexes fluoresce, and their fluorescence is red (Table V). (In this fluorescing group may also be included the complexes with 2 H<sup>+</sup>, 2 Na<sup>+</sup>, and 2 K<sup>+</sup>.)

An approximate notion of the firmness of the binding of the metals in the complexes may be obtained by making the metals compete with protons for the co-ordinating nitrogen atoms. The following metals are very firmly bound, as indicated by the fact that they are only displaced from the porphin in concentrated sulfuric acid: iron ( $\text{Fe}^{3+}$ ), copper ( $\text{Cu}^{2+}$ ), nickel ( $\text{Ni}^{2+}$ ), manganese ( $\text{Mn}^{3+}$ ), and cobalt ( $\text{Co}^{3+}$ ). Those displaced in strong hydrochloric acid are: zinc ( $\text{Zn}^{2+}$ ), tin ( $\text{Sn}^{2+}$ ), iron ( $\text{Fe}^{2+}$ ), and silver ( $\text{Ag}^{2+}$ ). The magnesium ( $\text{Mg}^{2+}$ ) and lead ( $\text{Pb}^{2+}$ ) are displaced from the complexes by dilute acetic acid. Instead of displacing firmly bound metals by high concentrations of protons, it is possible to reduce the metal porphin to a colorless porphinogen from which the metal is expelled. In this case, two of the six H atoms may be considered to have added to the N atoms, thus forming four pyrrole nuclei with no possibility of co-ordinating with the metal; *e.g.*, only by reduction can Cu be removed from *turacin*, the Cu uroporphin I. Another measure of the great firmness with which iron is held is by dissolving the iron porphin in a solution containing radioactive ferric ions and noting the rate at which iron exchanges. When this experiment was performed by Ruben *et al.* (112) no trace of exchange could be observed even after a period of two months.

In the dihydroporphins, the insertion of magnesium, for example, into pheophorbid, shifts the two strong bands (*i.e.*, the first and the ultra-violet) closer together, the first band being displaced about  $10\text{ m}\mu$  to shorter wave lengths and increased in height (Table III, page 346). If the acid form, or the copper complex of the dihydroporphins, is formed, a shift of the first band of  $20\text{ m}\mu$  and  $30\text{ m}\mu$ , respectively, to shorter wave lengths and an increase in height also occurs (121).

#### H. OPEN-CHAIN TETRAPYRROLES

A comparison of the absorption spectra of the mono-, di-, tri-, and tetrapyrroles brings out very clearly the effect of conjugation on the shape of their curves. (For the numbering of the substituents on the rings see Table VI on pages 360 and 361.)

##### 1. The Mono- and Dipyrroles

The absorption spectrum of a substituted pyrrole, *e.g.*, 2,4-dimethyl-3-ethylpyrrole-5-carbonic acid ester (*a*, page 359), consists of a single major band in the region  $250\text{--}300\text{ m}\mu$  (Fig. 13, curve *a*). When two pyrroles are attached as in dipyrromethane (*b*) or dipyrrolycarbinol, no conjugation exists between the rings, and the compound is colorless (*i.e.*,

absorbs in the ultraviolet); it has an absorption band in the same region as the monopyrrole, but because of the presence of two pyrroles the molar extinction ( $E = 28,000$ ) is about twice as high as for a single pyrrole ( $E = 13,500$ ) (Fig. 13, curve b). The presence of a keto group in the  $\alpha$ -position of a pyrrole, as for example, 5-pyrrolenone (d) displaces the maximum of the band to about 300  $m\mu$ . The presence of a hydroxy group in the  $\alpha$ -position of a pyrrole (for example, 5-hydroxypyrrole) depresses the extinction to about 3000.

When two pyrroles are conjugated through a methene link to form a dipyrromethene, the compound is yellow (for example, 3,5,3',5' - tetramethyl - 4,4' - diethyldipyrromethene—formula c) and has two bands in the region 200–500  $m\mu$ , the stronger band being at the longer wave length, for example, at 450  $m\mu$  with  $E = 20,000$  (Fig. 13, curve c). The substitution of hydroxy groups at the  $\alpha$ -positions, for example, 5,5'-dihydroxydipyrromethene (e) depresses the curve, the strong band being at 300  $m\mu$  and a weak afterband at 350  $m\mu$ .

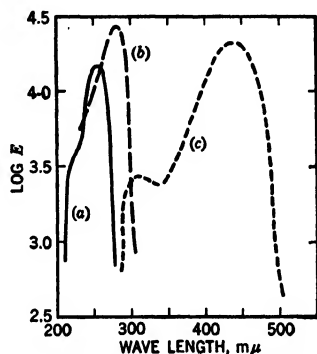
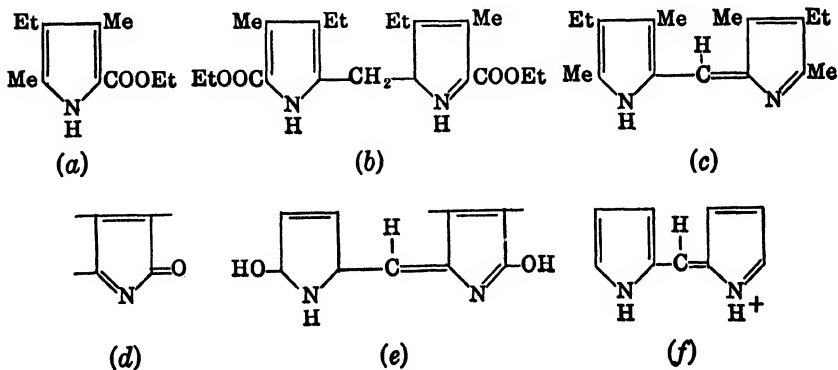


Fig. 13. Absorption curves for substituted pyrroles (102a).



The addition of a proton to the pyrrolenine nitrogen of a dipyrromethene creates a symmetrical resonating molecule (f) with an oscillating charge, the absorption being increased and shifted about 50  $m\mu$  to the visible

TABLE VI  
THE ABSORPTION SPECTRA AND COLOR TESTS OF THE OPEN-CHAIN TETRAPYRROLES AND DERIVATIVES

Compound	Formula*	Color		Absorption bands				Van der Berg + Pent- dione reaction	Ethio- chrome reaction	Zinc complex††	
		Neutral	Acid	Neutral	Acid	Neutral	Acid				
Pyrrole									+	+	
Pyromethane Bilirubin acid											
Pyromethene Alky!											

TABLE VI (Continued)  
THE ABSORPTION SPECTRA AND COLOR TESTS OF THE OPEN-CHAIN TETRAPYRROLES AND DERIVATIVES

Compound	Formula*	Color		Absorption bands				Van der Berg reaction	Fent- dycent reaction†	Ethlich reaction†	Zinc complex††	
		Neutral	Acid	Neutral		Acid					Absorption band wave length, mμ	Fluor-escence color
				λ, mμ	ε	λ, mμ	ε					
Bilidene		Yellow	Orange (HCl)	450	56,100 (CHCl <sub>3</sub> )			+	+	644,805 (1 <sub>2</sub> )	Red	
Bilirubin (Desmatodin)		Yellow	Orange	500 425	7,940 70,800			+	+	640,680 (1 <sub>2</sub> )	Red	
Mesobilirubin		Red	Red-violet (2 HCl)	585		605 557 497		0		625,510	Green-brown	
Mesobilirubin		Red-violet	Violet (2 HCl)	575		607 560		0		630,570	Red	
Biliverdine		Green	Green (3 HCl)	275 560 550	17,800 44,700 15,900			0	+	637 (1 <sub>2</sub> )	Red	
Glucobilin		Blue		309 353 535	17,800 45,000 15,000			0	+	630 (1 <sub>2</sub> )	Red	
Pterobilin		Green	Blue (3 HCl)					0		630 (1 <sub>2</sub> )		

Note to table: Molar extinctions are for dioxane as solvent (103) unless otherwise stated. Strongest bands of a series are underlined.

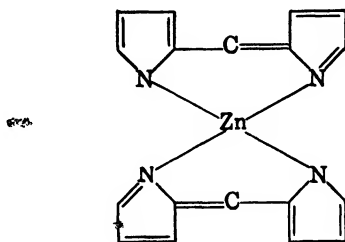
\* For side chain substituents of the naturally occurring biliverdins, see page 336; for substituent abbreviations, see Table IV, page 354.

†† Van der Berg reaction is the coupling at the free  $\alpha$ -position of pyrrole with diazotised sulfanilic acid. In biliverdins the splitting at the central methylene (CH<sub>2</sub>) bridge results in a free  $\alpha$ -position which can be coupled. The reaction is positive if a central methylene bridge is present and an OH is present on one or both ends.

† Ethrich reaction is given by numerous dipyrromethanes (also by biliverdins and heme compounds that can be oxidized by hydrogen peroxide to dipyrromethanes) which on subsequent reduction in alkaline solution become red and have an absorption band at 525 m $\mu$ .

†† Zinc salts are formed in alcoholic solution with zinc acetate. In some cases, oxidation with iodine, denoted by (1<sub>2</sub>), is required to form a colored zinc complex. Overoxidation produces green fluorescent compounds.

(Fig. 14). The formation of a dipyrromethene zinc "salt" (Fig. 14) (or a nickel, cobalt, or copper one) produces an effect similar to the addition of protons (if hydroxy groups are absent from the  $\alpha$ -positions). Such a zinc complex probably has a tetrahedral configuration (101) and may be represented as follows:



Almost all the dipyrromethenes fluoresce in solution and as solids (24). In neutral solution the dipyrromethenes fluoresce green to orange, many fluorescing with great intensity. In acid solution no fluorescence is observed. The solid dipyrromethenes, either as base or acid salts, and the solid zinc complexes of the dipyrromethenes fluoresce red, usually having a broad weak emission band at 590–640  $m\mu$ .

## 2. Tetrapyrroles—the Bilirubinoids

The relationships between absorption spectra and structures are shown in Table VI. In the bilanes (*e.g.*, mesobilinogen) no conjugation is present between the pyrroles, and these compounds are colorless and, like pyrroles, absorb in the ultraviolet.

When a methene bridge is present, as in the bilienes (*e.g.*, mesobilin) a conjugation occurs between two pyrroles and the spectrum resembles a dipyrromethene type. Just as with the dipyrromethenes, the addition of a proton shifts the two-banded spectrum about 50  $m\mu$  toward the visible and increases the intensity of absorption. When two dipyrromethene systems are present, separated by a methylene ( $\text{CH}_2$ ) bridge, as in bilirubin, the compound behaves as two separate dipyrromethenes with respect to its absorption, and the molar extinction is somewhat more than twice the value for a single dipyrromethene. Metals, like acids, also shift the spectra to longer wave lengths.

Three conjugated pyrroles, as in mesobilirhodin, shift the absorption well into the visible, and a broad band is present with a maximum at 580

$m\mu$ . In the bilitrienes, four pyrrole rings are conjugated through three methene bridges, as in biliverdin, and the biliverdin spectrum consists of two main bands, one at  $675 m\mu$  and a higher one at  $384 m\mu$  (Fig. 15).

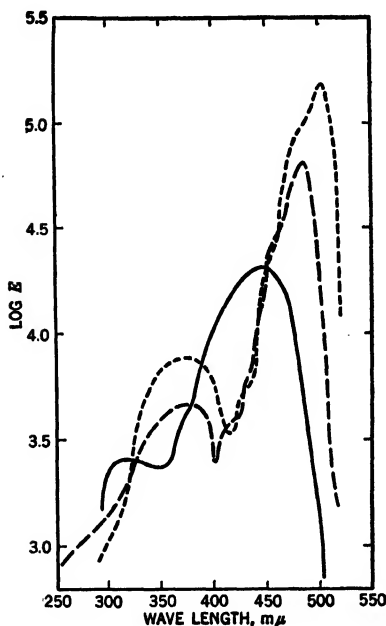
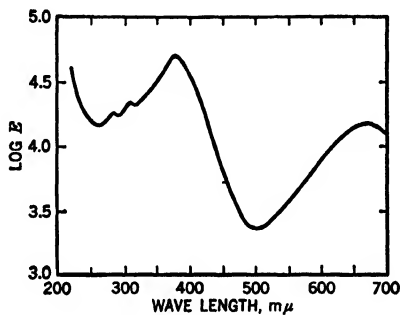


Fig. 14. Absorption curves (103) for 3,5,3',5'-tetramethyl-4,4'-diethylpyrromethene (—); hydrobromic acid salt (---); and zinc salt (— — —).

Fig. 15. Absorption curve of biliverdin dimethyl ester in chloroform (138).



According to Dh  r   (24) the tripyrroles as well as the bilirubinoid tetrapyrroles fluoresce. They have either one fluorescence band or a diffuse fluorescence in the visible, and in the powdered form they fluoresce red.

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# OXIDATION OF ORGANIC SULFUR IN ANIMALS\*

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## I. Introduction

The sulfur introduced into animal organs or tissues after ingestion and absorption is essentially present in organic linkage. It is therefore encountered in a number of different forms: as thio ester, as thiol, as disulfide,

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\* Translated from the French.

or in heterocyclic rings, and thus takes part in the make-up of molecules, the most important of which, quantitatively, are methionine, cysteine, and cystine. Aside from these three, which in a general way correspond to the greatest part of the ingested sulfur, there are other compounds, such as thiamine or biotin, found only in small quantities, which, however, play a fundamental role. Finally, there are other sulfur-containing substances which may occasionally be ingested and absorbed by the animals because they are food of animal origin on one hand, *e.g.*, taurine from the flesh of salt-water fish, and thiolhistidine (77) and ergothionine from the flesh of mammals, and of vegetable origin (120), on the other hand, *e.g.*, djenkolic acid isolated from *Pithecalobium lobatum* (123) and probably present in other legumes, the different isothiocyanates of *Cruciferae*, and the substances of which little is known as yet, and to which the potatoes owe their odor and taste (70). As far as inorganic sulfur is concerned, the only forms which must be considered are sulfates and sulfides. Sulfates are actually not ingested by animals, at least not by the higher nonmarine animals. If, in exceptional cases, the water consumed contains considerable amounts of sulfate which is but poorly absorbed by the intestinal tract, the small quantity of sulfate thus introduced into the organism is rapidly eliminated by the kidney without taking any part in metabolism. This inability of sulfate sulfur to enter the metabolic cycle of sulfur in the animal organism is particularly apparent in investigations with labeled sulfur. The latter, injected into rats in the form of sulfate, does not show up in the cystine stored by the animals (118). Regarding sulfides, they are rarely ingested by animals, but they frequently are formed in the digestive tract by bacterial action corresponding to desulfuration of cysteine, or to reduction of sulfate (149). Such sulfides are easily absorbed by the intestinal tract.

In the process of animal metabolism, the various molecules which contain sulfur serve different purposes: they may participate in the synthesis of more or less complex substances such as glutathione and proteins and thus serve in the formation of protoplasm or build up storage materials; they also may be destroyed immediately in more or less important degradation reactions. But all of them, sooner or later, finally undergo oxidation in varying degrees. The sulfur of such molecules thus is found again in a more or less oxidized state. It constitutes new molecules which may be eliminated in the urine, excreted with the bile, or, finally, stored in the course of metabolism in different organs or tissues. In the urine, especially of higher animals, the major part of the S is found as free or combined sulfate. It is also found, though in lesser quantities, as thio-sulfate (51), sulfocyanide, taurocarbamate, cystine, and oxyproteic acids (75), the latter made up for a large part of polypeptides with sulfonic acid groups, and for a less important part of polypeptides with disulfide linkage (76) to which urochrome is related. Sometimes, but again in very small quantity, sulfur in the urine may be en-

countered in the form of free taurine. Finally, sulfur in the urine is present in the form of oxidation products of thiamine and biotin. Sulfur is eliminated in the bile in the form of taurine combined with bile acids, *e.g.*, as taurocholic acid and others. In tissues and organs, aside from cystine, oxidized sulfur is found particularly in the following forms: free sulfate, *e.g.*, in the plasma (97); esterified sulfate forming a constituent of certain cerebrosides (20), mucoitin and chondroitin sulfuric acids which contribute to the structure of mucoproteins and of heparin (65); taurine in the muscles of salt water fish and molluscs, and in the adrenal glands of higher animals (93), asterubine in starfish (3); dimethyl sulfone in ox blood (101) and in adrenals (93); bis- $\beta$ -hydroxyethyl sulfide in adrenals (98,99); and, occasionally, adrenochrome, the heart pigment of *Paroctopus bimaculatus*, all the sulfur of which is in the oxidized state (41).

The oxidation reactions which the various forms of organic sulfur have undergone indicate a number of intermediary reactions. It is the aim of the present paper to study these reactions, their mechanisms, and the enzymic systems involved. The study is concerned with three points of view, namely, the action of enzymes on different sulfur-containing molecules *in vitro*, the oxidation of these molecules in the living animal, and the relations existing between the oxidation of organic sulfur and other aspects of general animal metabolism. In the following we shall consider first of all the oxidation of sulfur in the most important substances in sulfur metabolism of the animal: methionine, homocysteine, cysteine, and cystine. We shall then proceed to discuss the oxidation of hydrogen sulfide and of other less known and less important substances. On the basis of this we shall give a total picture in pointing out the relations which exist between the different oxidations, the result of which is manifested in the formation of the above mentioned oxidized sulfur compounds.

It is now appropriate, once and for all, to make two observations concerning the experiments with the living animal. (1) One sometimes observes differences in behavior of a substance to be oxidized, depending on whether that substance is introduced into the animal by ingestion or by injection. These differences are apparently due to complications resulting from microbiological action in the intestines on the ingested substance, before it had time to become absorbed. When there is a divergence between the results obtained in the course of experiments utilizing one or the other mode of introduction, it is evident that the only reliable data on the animal's metabolism are the results obtained after injection. Therefore this last method of introduction should be employed wherever possible. (2) If, after introduction of a sulfur-containing molecule into the organism, one notes an increase in the quantity of sulfur excreted in more or less oxidized state (sulfate, taurine, etc.), it is very probable that the sulfur thus elimi-



nated is the same as originally contained in the introduced molecule. But that is not always absolutely certain. In fact, one can think of certain cases in which the introduced molecule acts as a mobilizer for other sulfur-containing molecules, already present in the organism; in those cases it is the sulfur of the latter substances which is eliminated in oxidized form. The absolute proof that one can really identify the introduced with the eliminated sulfur can only be furnished by the use of labeled, radioactive sulfur,  $^{35}\text{S}$ . We shall deal further with the interesting applications of this element already frequently made.

## II. Oxidation of the Sulfur of Methionine

### A. OXIDATION OF METHIONINE SULFUR *IN VIVO*

The oxidation of methionine sulfur in the living animal appears to take place in both the *d*- and *l*-forms. Among the products of this oxidation, notably sulfate, thiosulfate, and taurine have been isolated.

*The oxidation of methionine sulfur to sulfate* in humans, after ingestion of the *l*-form was observed for the first time by Mueller (88). It has since been encountered in a number of other cases: in rabbits, after ingestion or subcutaneous or intravenous injection of the *dl*-form (138); in dogs after ingestion of the *dl*-derivative (94); and in humans after ingestion of either the *l*-, or the *dl*-form (84). This oxidation is quantitatively important, since the sulfate formed represents 60 to 70% (94) or even sometimes 100% of the introduced organic sulfur.

*The formation of thiosulfate* after injection of *dl*-methionine has been observed in rabbits (49) which were kept on an extremely low sulfur diet. Of the 378 mg. of methionine sulfur introduced, 0.6% was recovered from the urine after a 48-hour interval. This amount, though very small, is nevertheless significant. It bears out the fact that thiosulfate forms an intermediary compound in the oxidation of sulfur to sulfate, as we shall see on page 400.

Regarding *the oxidation of methionine sulfur to taurine*, the results of the experiments by Virtue and Doster-Virtue (134) with dogs have shown the existence of the oxidation reaction to be highly probable. The animals were first given a diet to deplete their taurine reserves; a bile fistula operation was performed; then ingestion of *dl*-methionine and cholic acid simultaneously was forced. This ingestion produces a decided increase in the quantity of excreted taurocholic acid. The absolute proof of the oxidation of sulfur of methionine to taurine has been furnished by Tarver and Schmidt

(119). These authors fed methionine containing radioactive sulfur to dogs and isolated from the bile of these animals taurine which contained the same sulfur. They state that the oxidation of methionine sulfur to taurine also takes place in rats.

Before we discuss the mechanism of these oxidation reactions it is appropriate to consider whether the integrity of the various characteristic groups of methionine, aside from the methiol group, is necessary for the oxidation of the sulfur of the molecule *in vivo*. It seems that no experiments have been carried out in which the carboxyl group of methionine was blocked. Regarding the amino group, Virtue and Lewis (138) have stated that if this group is blocked, *e.g.*, by the benzoyl radical, methionine sulfur can no longer be oxidized to sulfate. We shall see further on how this phenomenon must be interpreted.

## B. OXIDATION MECHANISMS OF METHIONINE SULFUR

### 1. Does Direct Oxidation of Methionine Sulfur Exist?

One of the first questions which one might ask in approaching the study of the mechanism of the oxidation of methionine sulfur in animals is whether this oxidation takes place immediately on the sulfur found in the initial molecule, thus furnishing as intermediary compounds methionine sulfoxide ( $\text{RSOCH}_3$ ) and methionine sulfone ( $\text{RSO}_2\text{CH}_3$ ). Regarding the oxidation to sulfate, the work of Virtue and Doster-Virtue (137) with dogs shows that the sulfur of methionine sulfoxide is less easily oxidized to sulfate than the sulfur of methionine. It appears therefore that methionine sulfoxide cannot be considered as an intermediary in the oxidation of methionine sulfur to sulfate, but that the methionine sulfoxide must rather previously be reduced to methionine, so that its sulfur can then be oxidized to sulfate. Regarding the formation of taurine, the work of the same authors shows that intravenous injection of methionine sulfoxide, preceding or accompanying the ingestion of cholic acid, produces an increase in taurine excreted in the form of taurocholic acid. It is thus possible that the sulfoxide might be an intermediary compound in the formation of taurine starting from methionine; but no absolute proof for this exists.

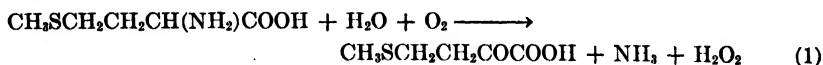
No work seems to have been done on the ability of methionine sulfone to furnish sulfate or taurine. We must simply point out that methionine sulfone cannot replace methionine in the diet of rats (8) and consequently is certainly not converted to methionine by this animal.

## 2. Oxidation of Methionine Sulfur after Previous Transformation of This Compound

Despite the lack of facts concerning the direct oxidation of methionine sulfur, it seems nearly certain that this direct oxidation plays no role in sulfate formation or contributes only negligibly to the formation of taurine. On the contrary, it appears that the oxidation of methionine sulfur takes place after previous conversion of the methionine to molecules of other types.

**Conversion of Methionine to  $\alpha$ -Keto- $\gamma$ -methiolbutyric Acid.** Methionine, as an amino acid, can undergo oxidative deamination to the corresponding  $\alpha$ -keto acid. In view of the importance of the stereochemical constitution of amino acids in this reaction, the case of the *d*- and *l*-configurations of methionine must be considered separately.

**Oxidation of *d*-Methionine by *d*-Amino Acid Oxidase.** The *d*-amino acid oxidase was discovered by Krebs, who extracted it from the liver and kidneys of rats (67,68) and who clearly distinguished it from *l*-amino acid oxidase (69). Warburg and Christian (142) have shown that the prosthetic group of this enzyme is alloxazine adenine dinucleotide, and Neglein and Brömel (89) obtained its protein portion in crystalline form. The oxidation of *d*-methionine by a preparation of *d*-amino acid oxidase which is more or less pure and free of catalase is accompanied by the formation of hydrogen peroxide (10). Bernheim and Gillaspie (12) have isolated and characterized the keto acid as its phenylhydrazone (m.p. 135°C.). The methionine undergoes an oxidative deamination reaction which, if one considers only its initial and final stages, corresponds to the equation:



This specific oxidation of *d*-methionine by *d*-amino acid oxidase has been utilized by Duschinsky and Jeannerat (37) for the preparation of *l*-methionine from the *dl*-derivative.

**Oxidation of *l*-Methionine by *l*-Amino Acid Oxidase.** Two enzyme systems exist which can bring about specific oxidative deamination of *l*-methionine: *l*-amino acid oxidase of the tissues of higher animals, and ophio-amino acid oxidase of the venom of serpents.

The *l*-amino acid oxidase which acts on *l*-methionine, was isolated from the liver and kidneys of rats by Blanchard, Green, Nocito, and Ratner (16,53). Its electrophoretic behavior indicates that this enzyme exists in two homogeneous forms (17), each of which contains a prosthetic group

made up of riboflavin phosphate. From the flavin content of both forms, it appears that one of them—the lighter—contains two flavin molecules per molecule of enzyme, whereas the other heavier one contains eight. The  $\alpha$ -keto- $\gamma$ -methiolbutyric acid produced by the action of this *l*-amino acid oxidase on *l*-methionine has been isolated and characterized in the form of its 2,4-dinitrophenylhydrazone (m.p. 149°C.) (53).

The ophio-*l*-amino acid oxidase was discovered in the venom of *Vipera aspis*, *V. libetina*, *V. latastei*, *Borthrops atrox*, *Naja* sp., etc. and was studied by Zeller and Maritz (146,147). It is differentiated from *l*-amino acid oxidase of tissues of higher animals by its inability to oxidize *l*-proline, *l*-oxyproline, and *N*-methyl-*l*-leucine and its insensitivity toward ammonium sulfate (148). Otherwise, it produces the same oxidative deamination reactions and acts particularly on *l*-methionine.

The oxidative deamination of *d*- and *l*-methionine also takes place *in vitro* with tissues slices (kidneys and liver of rats) (67,140), kidney slices showing a greater activity than liver slices. In both cases, but especially with liver slices, the reaction is enhanced by arsenic trioxide (22). This reaction takes place *in vitro* and in the intact animal alike. The  $\alpha$ -keto- $\gamma$ -methiobutyric acid actually can be isolated in the form of its dinitrophenylhydrazone from the urine of rats after the animals were fed a carbohydrate diet with added methionine (139).

It thus appears that in animals *d*-methionine as well as *l*-methionine can furnish the corresponding keto acid by oxidative deamination. But it is actually impossible to state there exists a relationship between the similarity in behavior of the two stereoisomers and the similarity of their ability to form sulfate. The fate of sulfur of the  $\alpha$ -keto- $\gamma$ -methiobutyric acid is little known as yet; according to Waelsch and Borek (140) the acid is broken down in acid or alkaline medium or by boiling to methyl mercaptan and an undetermined residue, and the authors think that this possibly represents a model of a physiological reaction. On the other hand, this same acid seems to be able to take part in the same transmethylation reactions as *l*-methionine (58). It is therefore very unlikely that the sulfur of this keto acid can be oxidized directly to sulfate.

**Conversion of Methionine to Homocysteine.** The conversion of methionine to the corresponding keto acid does not bring the thiomethyl group directly into action. On the contrary, the fact that this group plays the principal role in the demethylation of methionine has been demonstrated in the work of du Vigneaud and collaborators. Numerous observations have been carried out with living animals which have shown

the close relationship between methionine and homocysteine. These relations imply that the latter substance is formed by demethylation of the former.

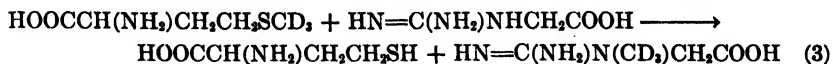
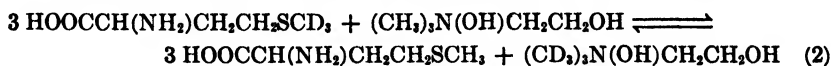
It is in this manner that diets deficient in sulfur-containing amino acids which may consist in part of arachine (144) or in part of conveniently treated casein (129) and thereby inadequate for growth of young rats, regain this ability if one adds homocystine or methionine, much better than if one adds cystine. In the case of such diets, homocystine replaces methionine perfectly, and it is evident that under more or less defined conditions, it can easily be reduced in the organism to homocysteine (35). The *d*- and the *l*-homocystine can be utilized equally well for the growth of the animal. This behavior is analogous to the action of *d*- and *l*-methionine (38). On the other hand, if one feeds methionine to rats or rabbits, a substance appears in the urine which gives a positive cyanide-nitroprusside test (29). This substance is not cystine, but has been considered to be homocystine (138). Finally, according to Medes (83), human urine should normally contain small quantities of homocystine. But it was later shown, that homocysteine is not equivalent to methionine in animal metabolism. Du Vigneaud, Dyer, and Kies (130) as well as Rose and Rice (100) made the statement in 1939 that, in certain types of well-defined diets, homocystine cannot replace methionine in maintaining growth of animals which were fed a diet lacking in cystine and methionine. There thus exists a supplementary factor which must accompany homocysteine and which is necessary for the conversion of homocystine or homocysteine to methionine, in other words necessary for the methylation of homocysteine. Du Vigneaud and co-workers (127) found this factor to be choline. It appears thus that choline acts as donor of the methyl group necessary for the synthesis of methionine from homocysteine.

Higher animals therefore are themselves unable to produce the methyl groups necessary for this type of methylation and for other types. The methyl groups must thus be present in the diet in a particularly labile form as in choline or methionine. A reversible exchange of methyl groups must exist between choline, methionine and possibly other substances. Good illustrations for this view have been presented by du Vigneaud and collaborators (126,128). They have in effect demonstrated that, in the case of rats, transport of the methyl group of methionine makes possible the synthesis of choline and creatine. This demonstration is based on the isolation of deuteriocholine and deuteriocreatine, obtained from the urine of rats which had been fed a diet with methionine containing deuterio-

methyl groups. From the deuterium content of the isolated substances, it appears that, after a certain time, each of the substances in question contains about the same percentage of methyl groups of the deuteriomethionine of the diet. For a duration of 14 weeks, the concentration of deuterium in the methyl groups of the three compounds was 85% of the methionine content which shows that practically no compound in the diet other than methionine furnished the methyl groups. The trimethylamine obtained by oxidation with alkaline permanganate shows that all deuterium is localized in the methyl groups of choline. Furthermore, the role of methionine as a transport agent for methyl groups has likewise been demonstrated by the same authors. This was done by isolation of deuteriocreatine and deuteriocreatinine from animals fed a diet containing deuteriocholine and homocystine.

Simmonds and du Vigneaud (108) have also demonstrated the utilization of the methyl group of methionine for synthesis of choline and creatine in man. Similar experiments (104) have shown the existence of the same reaction in the synthesis of creatine, choline, and anserine in the rabbit. Finally, using deuterium, Simmonds, Cohn, Chandler, and du Vigneaud (107) have shown the possibility of methyl group transfer from choline to methionine in the rat. There thus comes about the concept of biologically labile methyl groups which plays a fundamental role in the sulfur and nitrogen metabolism. Its importance has recently been emphasized by du Vigneaud (124). It is today well established that the exchange of methyl groups between methionine and choline is reversible, although the transfer of methyl groups from methionine or choline to guanidoacetic acid, with the formation of creatine, is irreversible. This role of methionine as a donor of methyl groups explains the older observations of Stekol and Schmidt that the quantity of urinary creatine increases in dogs after ingestion of *dl*-methionine (116).

These results may be shown schematically in the following equations:



These transmethylation phenomena have also been studied *in vitro* with tissue slices. Borsook and Dubnoff (23) have thus ascertained the formation of creatine, starting with glycocyamine by methyl transfer from *dl*-methionine, with rat liver slices.

It is of interest to point out the difference in behavior of the stereoisomers of methionine. The work of Handler and Bernheim (58) shows in effect that *d*(+)-methionine has only 50% of the activity of the natural isomer in the synthesis of creatine by rat liver slices. Besides, the transmethylation with *d*(+)-methionine is inhibited by benzoic acid, while the latter has no action on the transmethylation with *l*(-)-methionine. Furthermore, benzoic acid inhibits the action of *d*-amino acid oxidase, it appears that the transmethylation observed in the presence of *d*(+)-methionine does not proceed directly from that compound, but, only after its deamination, from the corresponding keto acid.

The importance of the demethylation on the oxidation of methionine to sulfate is borne out particularly by the work of Borek and Waelsch (22). These authors repeated quantitatively the studies by Borsook and Dubnoff and found that under their experimental conditions only 2% of the introduced methionine is utilized for methylation of glycocyamine. This 2% corresponds quite reasonably to the quantity of sulfate formed. This agreement is an argument in favor of the hypothesis that methionine sulfur cannot be oxidized to sulfate unless its methyl group is previously accepted by another compound. Furthermore, this oxidation can only take place if an acceptor for the sulfhydryl group exists in the system.

### III. Fate of the Sulfur of Homocysteine

The formation of homocysteine from methionine is thus an established fact. This homocysteine, in animals, and particularly in dogs, undergoes a series of reactions, resulting in the oxidation of its sulfur and excretion of the latter in the form of sulfate or taurine (136). We are thus led to the study of the mechanisms of this oxidation.

#### A. DIRECT OXIDATION OF THE SULFUR OF HOMOCYSTEINE

##### 1. *Oxidation of Homocysteine to Homocystine and the Fate of Homocystine*

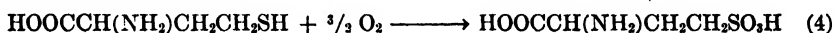
In analogy with what is known about the oxidation of cysteine to cystine, one can admit the possibility of the oxidation of homocysteine to homocystine, under certain conditions, although no exact study of such an oxidation by a biological path seems to have been carried out as yet. It is of interest to compare homocysteine metabolism with that of homocystine. In comparing these two, one notes very distinct differences in the metabolism. The work of Virtue and Doster-Virtue (134) shows that the sulfur of homocystine ingested by dogs is excreted mainly in the form of

sulfate, with practically none in the form of taurine. Furthermore, a study of a case of cystinuria in man by Brand, Cahill, and Block (26) indicates that nearly all of the ingested homocystine sulfur was oxidized to sulfate, while about 50% of the sulfur of homocysteine, like the sulfur of cysteine and methionine was excreted in the form of extra cystine, with 27% only in the form of sulfate, and about 24% in the form of homocystine. In addition, homocystine is distinguished *in vitro* from homocysteine by its inability to undergo a desulfuration reaction, which will be discussed below. Therefore, homocysteine as well as homocystine are not always equivalent in animal metabolism. It is always possible that in the organism, at least a portion of the homocystine which would eventually have been formed from homocysteine can be reduced to that compound. The metabolism of homocystine can therefore follow two paths: homocystine can be reduced to homocysteine, thus following the fate of the latter or by an oxidation mechanism peculiar to it, and as yet unknown, homocystine can finally yield sulfate.

It should be pointed out that neither homocysteine nor homocystine can be deaminated to the corresponding keto acids (22).

## 2. Oxidation of Homocysteine to Homocysteic Acid

Medes and Floyd (86) have shown that cysteine oxidase B, the action of which will be discussed later, acts also on homocysteine and oxidizes it to homocysteic acid:



One actually does not know whether this enzymic reaction plays a role in homocysteine metabolism in animals.

## B. DESULFURATION OF HOMOCYSTEINE WITH LIBERATION OF HYDROGEN SULFIDE

The sulfur of homocysteine can be split from organic molecules in the form of hydrogen sulfide. This desulfuration reaction is due to the presence of an enzyme, homocysteine desulfhydrase, which was discovered by Fromageot and Desnuelle (45). This enzyme is found in the liver, the pancreas, and the kidneys of higher animals. It can be distinguished clearly from another enzyme, cysteine desulfhydrase, which we will encounter on page 390, by the fact that the amounts of hydrogen sulfide liberated from cysteine and homocysteine, by different enzyme preparations, vary in proportion. It is possible, for example, in starting with an aqueous ex-



tract of an organ, *e.g.*, liver, to observe no activity on homocysteine, yet to conserve all activity toward cysteine. The homocysteine sulphydrase can also be distinguished from cysteine desulphydrase by its greater instability, which up to now has been an obstacle in the purification of the enzyme and which consequently has prevented the convenient study of the complete reaction which it produces. The behavior of the *d*- and *l*-derivatives of homocysteine differs considerably with regard to susceptibility to the action of desulphydrase. This enzyme displays marked stereochemical specificity, the velocity of desulfuration of *d*-homocysteine being on the average about 15% of the ~~velocity~~ velocity of the *l*-form (34). The path of the sulfur of hydrogen sulfide thus liberated will be discussed on page 397.

#### C. DESULFURATION OF HOMOCYSTEINE BY TRANSSULFURATION

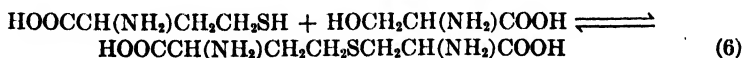
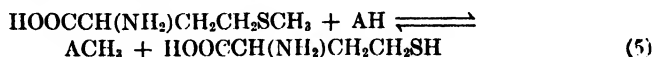
The sulfur of homocysteine can be transferred to serine in a trans-sulfuration reaction which thus produces cysteine. The evidence for this important reaction was furnished by the work of du Vigneaud and collaborators. Numerous observations have shown the possibility of cysteine formation from methionine in animals. In this manner, according to Beach and White (6), rats fed a diet practically devoid of cystine, but supplemented with methionine, toward the end of the experiment stored a quantity of cystine in their tissues. This amount by far exceeded the little cystine ingested and the cystine present at the beginning of the experiment. Dawbarn (32) likewise has stated that the quantity of cystine stored in the hair of rats fed a diet essentially free of cystine but supplemented with methionine is by far larger than the amount ingested by the animals in the course of the experiment. Finally the work by Tarver and Schmidt (118) and by du Vigneaud, Kilmer, Rachele, and Cohn (131) shows that the sulfur of cystine extracted from rats which had been previously fed methionine with radioactive sulfur, is radioactive itself. The proof of the above-described transmethylation being established, these observations can also actually be considered to pertain to the formation of cystine from homocysteine.

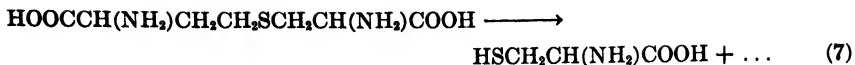
Other observations show the existence of such a transformation beyond any doubt. Such is the work by Brand, Cahill, and Block (26) cited above, according to whom the sulfur of homocysteine ingested by a patient suffering from cystinuria is excreted for the largest part in the form of extra cystine.

Among the theories proposed to explain the formation of cysteine from homocysteine, the one by Brand, Block, Kassell, and Cahill (25)

is of particular interest. These authors were in effect the first to postulate that the formation of cysteine from homocysteine implies the transport of sulfur from a  $C_4$  to a  $C_3$  chain, with a transitory formation of *S*-( $\beta$ -amino- $\beta$ -carboxyethyl)-homocysteine. The validity of this assumption was confirmed in a brilliant manner by the work of du Vigneaud and co-workers, who have precisely stated the nature of the  $C_3$  compound which acts as acceptor of the sulfhydryl group. This compound is not aminoacrylic acid—as had been postulated by Brand *et al.*—but serine. Du Vigneaud, Brown, and Chandler (125) have synthesized *l,l*-*S*-( $\beta$ -amino- $\beta$ -carboxyethyl)-homocysteine, a mixed thio ester consisting of  $\alpha$ -alanine and  $\alpha$ -aminobutyric acid, to which they gave the name cystathionine. They have stated that this compound can maintain the growth of rats fed a diet free of cysteine and containing only methionine in suboptimal amount. Cystathionine can otherwise not replace homocysteine. On the other hand, Binkley, Anslow, and du Vigneaud have shown in a subsequent paper (14), that rat liver slices and even extract of liver in solution can form cystine from cystathionine. These authors were able to obtain in five hours an amount of cystine equal to about 30% of the theoretical. The reaction is favored by anaerobic conditions, under which it produces hydrogen sulfide, obviously due to the action of cysteine desulfhydrase on the cysteine formed. Sodium cyanide inhibits this hydrogen sulfide evolution without hindering the transformation of the thio ether to cysteine. Somewhat later, Binkley and du Vigneaud (15) succeeded in demonstrating the nature of the intermediary compound—cystathionine—in the cysteine formation starting with homocysteine. With *dl*-homocysteine and *dl*-serine in the presence of rat liver slices, or soluble liver extract under anaerobic conditions for several hours, these authors were able to verify the formation of cysteine. They obtained 3.6 mg. of the latter compound, starting with 10 mg. of homocysteine. The optical specificity of this reaction is very marked, since *d*-homocysteine and *d*-serine do not give rise to cysteine. The production of cysteine takes place also in the absence of serine, but to a much lesser degree.

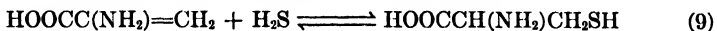
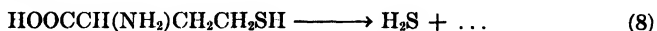
The over-all results of the work of du Vigneaud and his collaborators, describing the synthesis of cysteine from methionine sulfur in higher animals, is shown in the following scheme:





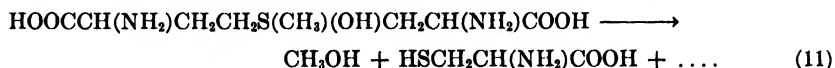
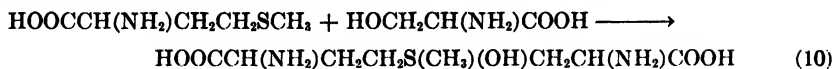
The validity of this mechanism is further confirmed by the observations of Stetten (117), who fed rats a diet containing serine with isotopic nitrogen,  $^{15}\text{N}$ , and found that after three days the cystine in the tissue proteins is particularly rich in labeled nitrogen, thus furnishing the proof for the transfer of the carbon chain from serine to cystine.

The methionine sulfur is finally transformed into cysteine sulfur. Before studying the latter substance and the oxidation of its sulfur, one must ask whether there are no other mechanisms for the formation of cysteine from methionine. Among the theories proposed on this subject, one by Brand, Block, Kassell, and Cahill (25) interposes  $\alpha$ -aminoacrylic acid and not serine as the sulfur acceptor for homocysteine in the process of transsulfuration. Binkley and du Vigneaud (15) have stated that, according to their experience, serine cannot be replaced by a mixture of pyruvic acid and ammonia. This mixture, according to a reaction leading to an equilibrium, produces aminoacrylic acid. It follows that the theory of Brand and his collaborators regarding the role of this acid in the formation of cystathionine should be abandoned. On the other hand, the function of homocysteine desulfhydrase and of cysteine desulfhydrase (45), the formation of  $\alpha$ -aminoacrylic acid from cysteine by the action of cysteine desulfhydrase (111), the ability of the latter enzyme to act reversibly (114), and the possibility of a reaction between  $\alpha$ -aminoacrylic acid and hydrogen sulfide (91) lead one to envisage the possibility of the following reaction mechanism:



The action of cysteine desulfhydrase is inhibited by hydrogen cyanide. Binkley and du Vigneaud (15) have observed that the production of hydrogen sulfide, which appears in the course of the reaction due either to homocysteine desulfhydrase or to cysteine desulfhydrase, is arrested in the presence of hydrogen cyanide, without however diminishing appreciably the yield of cysteine. This observation shows that the mechanism in question does not come into play in the experiments of Binkley and du Vigneaud, but the results do not show that this mechanism plays no role in the animal. The theory of Toennies (121) assumes the existence of a direct transsulfuration between methionine and serine. This theory is based on the ability of methionine sulfur to form sulfonium derivatives

under the contingent participation of hydroxyamino acids. This ability may be shown schematically in the following equations:



The validity of this theory has neither been confirmed nor denied by the experiment. It is possible that the mechanism conceived by Toennies plays a role in the formation of substances such as the methyldiethylsulfonium ion of the urine of dogs (90). But whatever the mechanism describing the formation of cysteine from methionine, it always concerns exclusively the transfer of the methionine sulfur to a  $\text{C}_3$  chain. There was never a fragment of a  $\text{C}_4$  chain of methionine encountered in cysteine, as had been supposed formerly. This conclusion rests on the experiments of du Vigneaud, Kilmer, Rachele, and Cohn (131), who actually synthesized methionine with the carbon isotope,  $^{13}\text{C}$ , in the  $\beta$ - and  $\alpha$ -positions and with radioactive sulfur,  $^{34}\text{S}$ . They fed this methionine to rats. After about 35 days, the animals were shorn as closely as possible and their hair was analyzed for cystine. It was found that about 80% of the sulfur of the cystine came from the ingested methionine, although the same cystine did not contain any carbon of the ingested methionine. This is irrefutable proof that the carbon chain of methionine is not utilized *in vivo* in the formation of cysteine from methionine.

#### IV. Metabolism of Cysteine

Cysteine thus appears both as a primary substance of animal metabolism, and as an intermediary substance in the transformations of the sulfur initially furnished by methionine.

##### A. OXIDATION OF CYSTEINE SULFUR *IN VIVO*

In contrast to the fact that *l*-cysteine alone can be utilized for growth, oxidation in the living animal of the sulfur from *d*- or *l*-cysteine seems to proceed equally well. Among the products resulting from this oxidation cystine, sulfate, thiosulfate, and taurine have been identified.

The oxidation of cysteine to cystine manifests itself in a particularly striking fashion in individuals suffering from cystinuria. Brand, Cahill, and Harris (27) have stated that 70% of the ingested *l*-cysteine sulfur was found in the urine of a cystinuric in the form of cystine. In the normal

animal there is certainly the possibility for the transformation of cysteine into cystine (84) as otherwise for the reverse reaction. In spite of that, it is equally certain that the metabolism of these two amino acids differs, at least in certain cases. The study of the oxidation of the sulfur which they contain will be treated here separately.

*The oxidation of cysteine sulfur to sulfate* is a well-established fact. Hele and Pirie (59), in experiments with dogs, stated that, in the two days following its ingestion, about 70% of the sulfur of cysteine is excreted in the urine in the form of sulfate, the result being practically the same whether one uses *l*- or *dl*-cysteine. Medes, in experiments with man, has pointed out (84) that the oxidation of cysteine sulfur to sulfate can account for 73 to 95% of the total sulfur, depending on whether it was introduced by injection or ingestion. Finally, in the case of rabbits, the intravenous injection of *l*-cysteine also produces a pronounced increase in urinary sulfate (49). Further experiments have been carried out to establish at which point the blocking of the different characteristic functions of cysteine may influence the oxidation of sulfur to sulfate in the animal. These results are shown in Table I.

TABLE I

OXIDATION OF SULFUR OF VARIOUS *l*-CYSTEINE DERIVATIVES TO SULFUR IN ANIMALS\*

Amino acid	Animal and path of introduction	Sulfate formed	Ref. No.
Acetylcysteine	Dog, subcutaneous injection	+	59
Glycylcysteine	Dog, subcutaneous injection	+	59
Reduced glutathione	Dog, subcutaneous injection	++	59
Chloracetylcysteine	Dog, subcutaneous injection	0	59
<i>S</i> -Carboxymethyl- cysteine	Rabbit, subcutaneous injection	0	21
<i>S</i> -Methylcysteine	Rabbit, ingestion	++	132
	Normal man, ingestion	++	24
	Man suffering from cystinuria, ingestion	+	31

\* ++, same order of magnitude as in the case of cysteine introduced under the same conditions. +, considerable but decidedly less than under ++. 0, no sulfate formed.

*The oxidation of cysteine sulfur to thiosulfate* has been observed in rabbits after intravenous injection of *l*-cysteine. Of 338 mg. of *l*-cysteine sulfur introduced, 0.8% was recovered in the urine in the form of thiosulfate after a 48-hour interval (49). As in the case of methionine, this amount is very small; its slightness is explained in the same way. However, the amount is significant.

For *cysteine sulfur oxidation to taurine*, it is curious that we must state that to the best of our knowledge there are no direct experimental facts concerning such an oxidation *in vivo*. However, the experiments *in vitro* with enzyme preparations make the oxidation of cysteine sulfur to taurine in animals more probable, as we shall see later on.

## B. OXIDATION MECHANISMS OF CYSTEINE SULFUR

### *Direct Oxidation of Cysteine Sulfur*

Contrary to what takes place in the case of methionine, the direct oxidation of the sulfur of cysteine plays a very important role in the metabolism of this compound. This oxidation leads, depending on conditions, to cystine, cysteinesulfenic and cysteinesulfinic acids, or to the corresponding sulfonic acid, cysteic acid.

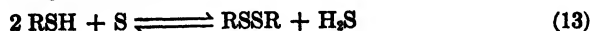
**Oxidation to Cystine.** The types of oxidation of cysteine to cystine differ depending on the nature of the hydrogen acceptor in the system: oxygen, sulfur, aminoacrylic acid, pyruvic acid, etc. The oxidation of cysteine in the presence of aminoacrylic acid and pyruvic acid will be discussed later in conjunction with the action of cysteine desulfhydrase.

*Oxidation in the Presence of Oxygen.* One knows from Sakuma's experiments (102) that, contrary to what was believed previously, carefully purified cysteine in neutral solution, completely free of traces of metals, is practically not oxidized by oxygen from air. On the contrary, this oxidation is very strongly catalyzed by iron, copper, and manganese (71,102, 141,143) even if the metals present are traces. In animal tissues, the role of oxidation catalyst for cysteine is accomplished by cytochrome oxidase in the presence of cytochrome c. The action of this system on cysteine has been studied in detail by Keilin (66) and by Medes (85), and is represented by:



This action is inhibited by hydrogen cyanide, sodium azide, and carbon monoxide. The inhibition by carbon monoxide is suppressed by light (66). It should be pointed out that cytochrome oxidase, with or without cytochrome c, does not exert any oxidizing action on methionine or cysteine sulfinic acid.

*Oxidation in the Presence of Sulfur.* Cysteine in the presence of finely divided or colloidal sulfur is oxidized to cystine, and, at the same time, sulfur is reduced to hydrogen sulfide:



This reaction has been utilized by Smythe (111) for the determination of the sulfur formed in the tissues by the reverse reaction. This hydrogen exchange between cysteine and sulfur is important from a biological viewpoint, as a consequence of the simultaneous presence of hydrogen sulfide, cystine and cysteine in the tissues. This point will be discussed in detail later.

Aside from the special cases which have been reviewed, it is evident that cysteine can act as hydrogen donor toward the most diversified hydrogen acceptors, provided that the respective redox potentials are favorable and that the enzymes necessary for the corresponding hydrogen transfer are present. In the work of Fraenkel-Conrat and collaborators (42), a bibliography is given of proteins (enzymes, hormones, etc.) in which the disulfide groups are reducible to sulfhydryl groups, coupled to the oxidation of cysteine to cystine.

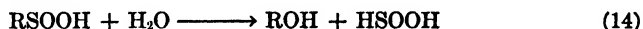
#### **Oxidation to Sulfenic and Sulfinic Acids, and Fate of These Acids.**

Pirie (95) used slices of liver and kidneys of rats and found that these tissues oxidize the cysteine sulfur to sulfate. Having thus shown the enzymic character of this oxidation, he attributed it to the existence of a "cysteine oxidase." This substance is lacking in blood, testicles, spleen, heart, and lung. The same enzymic reaction was observed by Medes (85) with a filtrate of ground rat liver in a solution buffered with carbonate. The enzymic system under observation, suspended either in a carbonate buffer solution, or in a dilute neutral salt solution, is easily adsorbed on permutite, but its subsequent elution has not been accomplished to date. Otherwise, the same enzymic system is precipitated upon addition of magnesium or sodium sulfate, but it then loses all its activity.

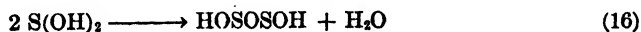
It is obvious that the oxidation of cysteine sulfur to sulfate is not a simple reaction. This oxidation implicates intermediary reactions some of which at least necessitate the intervention of a particular enzyme. The term "cysteine oxidase"—replaced by Medes and Floyd (86) with "cysteine oxidase A" in order to differentiate the enzymic system oxidizing cysteine sulfur to sulfate from the one oxidizing cysteine to cysteic acid, "cysteine oxidase B"—must be considered as a summary expression that groups together enzymes which in their actions correspond to simpler elementary transformations. The nature and the role of the possible intermediaries in the oxidation of cysteine sulfur to sulfate have been discussed by Medes and Floyd (86). According to these authors, the peculiar action of cysteine oxidase A consists in the oxidation of cysteine to cysteinesulfenic acid,  $\text{RSOH}$ . This acid is unstable under physiological conditions.

In a spontaneous dismutation reaction between two molecules of this acid, one molecule of sulfinic acid is created and at the same time one molecule of cysteine is regenerated. The sulfinic acid thus formed can serve two different purposes. First, it may bind an additional oxygen atom and thus form cysteic acid. This oxidation would conform with the ability of the sulfinic acid to produce taurine, which was experimentally demonstrated in dogs by Virtue and Doster-Virtue (135). Second, the sulfur of the cysteinesulfinic acid may be oxidized to sulfate which would entail a fission of sulfur from the carbon chain. The mechanism of the fission and of this oxidation has not been completely elucidated.

A primary experimental fact relating to this reaction is the formation of sulfate (under aerobic conditions) from cysteine sulfinic acid by the action of ground or extracted rat livers (85). This oxidation was carried out by Medes (85) in order to study the existence of a sulfinic acid oxidase responsible for the action. But it is difficult to assume that a single enzyme would have the ability to split the cysteinesulfinic acid into an organic molecule without sulfur and into inorganic sulfur, and then oxidize the latter to sulfate. Numerous explanations have been proposed to resolve this difficulty. According to Pirie (95), the removal of the sulfur from cysteine-sulfinic acid would take place as sulfite, which in turn would be oxidized spontaneously to sulfate (reaction 31). Medes and Floyd (86) have two other theories to explain the sulfate formation. The first of these two theories may be written:



The thiosulfate thus formed is in turn easily oxidized to sulfate (150). This theory is interesting since it could furnish one of the explanations for the constant presence of thiosulfates in the urine of higher animals (51). The second theory of Medes and Floyd is based on the conversion of sulfoxylic acid to disulfoxylic acid in alkaline solution (5).



The disulfoxylic acid then reacts in turn with a second molecule of sulfoxylic acid to form pyrosulfuric acid:



which is known to be easily converted to sulfate.

Other facts demonstrate the complexity of the reaction (48): cysteine-



sulfinic acid, injected subcutaneously in the form of its sodium salt into rabbits, produces a considerable increase in the amount of thiosulfate excreted in the urine. One might think at first that this constitutes an argument in favor of the validity of reactions (14) and (15). But Fromageot *et al.* (48,50) have recently furnished the proof for the existence of an enzyme in the liver of rabbits, desulfinicase; its action is displayed by the liberation of sulfurous acid (or sulfur dioxide) under anaerobic conditions from *l*-cysteinesulfinic acid, in conformity with Pirie's scheme. These results lead only to hypotheses: one could suppose first of all, that different organs contain different enzymic systems, some acting according to equations (14) and (15) and forming thiosulfate, and other, such as the liver, which create sulfite. Sulfite, like thiosulfate, is easily oxidized in the organism to sulfate (105). One might also suppose that the thiosulfate results from a reaction of sulfite with sulfur coming, as shown on page 391, from the action of cystine on hydrogen sulfide, the latter being formed by desulphydrases. It is thus actually impossible to state precisely the intermediary steps of the oxidation of sulfinic acid sulfur to sulfate.

**Oxidation to Cystic Acid and Fate of This Acid.** Bernheim and Bernheim (11) were the first to state that cysteine, in the presence of ground rat liver extract under anaerobic conditions at pH 6.7, binds three oxygen atoms per molecule, thus forming, in all probability, cystic acid,  $\text{HOOCCH}(\text{NH}_2)\text{CH}_2\text{SO}_3\text{H}$ . The system which catalyzes this reaction is thermolabile. It is inhibited at pH 6.7 by sodium pertitanate. Medes (85) and later Medes and Floyd (86) studied this oxidation of cysteine to cystic acid, using ground rat liver which had been washed several times by centrifugation and successively suspended in a 0.01 *M* phosphate buffer solution at pH 6.7. After verifying that the reaction corresponds well to the fixation of three oxygen atoms by a cysteine molecule, the authors have pointed out that the inhibition of the oxidation by sodium pertitanate which occurs at pH 6.7 does not take place at pH 7.5. The enzymic system in question, cysteine oxidase B, reacts as well with *dl*-cysteine and with the *d*-derivative. It is hardly probable that the oxidation of cysteine to cystic acid takes place directly. Among the possible intermediates one might consider sulfinic acid. In fact, experiments carried out by Medes (85) show that the enzymic preparation which oxidizes cysteine to cystic acid likewise oxidizes cysteine sulfinic acid to cystic acid, with about the same velocity. The problem whether it is a single enzyme which is capable of oxidizing cysteine and cysteine sulfinic acid, or two different enzymes, has not yet been completely elucidated. But this observation makes the

role of the sulfinic acid as an intermediate in the above-described oxidation more probable.

Cysteic acid appears thus as a physiologically important stage in cysteine oxidation, and this leads us to the study of the fate of this acid. The observations of Schmidt and Clark (105) bear upon ingestion of cysteic acid by dogs; those of White, Lewis, and White (145) deal with ingestion and parenteral injection of the same substance in rabbits; those of Medes (84,87) are concerned with ingestion in man. They all show that, depending on the mode of introduction, cysteic acid produces or does not produce an increase in urinary sulfate. No increase is observed after injection, which shows the evident intervention of the intestinal bacterial flora in the formation of sulfates. The fact that injection of cysteic acid does not produce an increase in the amount of sulfate excreted by the animal shows that cysteic acid is not an intermediate in the cysteine oxidation to sulfate. Cysteic acid, however, undergoes two types of degradation in higher animals, namely, a decarboxylation reaction furnishing taurine, and an oxidative deamination reaction which produces the corresponding keto acid, *i.e.*, sulfoypyruvic acid.

The work by Virtue and Doster-Virtue (135) has shown that formation of taurine takes place. These authors experimented with dogs which were properly starved and provided with bile fistulas. They introduced cholic acid and at the same time cysteic acid by ingestion and concluded that formation of taurocholic acid was unquestionable. Such a conversion of cysteic acid to taurine obviously implies a decarboxylation:

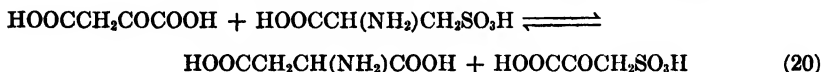
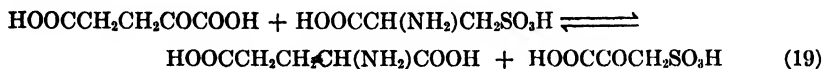


The enzyme responsible for this action was discovered by Blaschko (18) in the liver of dogs and rats. This enzyme, cysteic acid decarboxylase, is specific for *l*-cysteic acid. It is inhibited by prolonged dialysis and partially inhibited by *dl*-homocysteic acid on which it has no action (19).

Medes and Floyd (87) studied the action of different tissue slices in the presence of air and ascertained the presence of cysteic acid decarboxylase in spleen, but, in contrast to Blaschko's findings, did not find it in the liver, heart, or muscles. The decarboxylating and deaminating activities of the intestinal mucosa appear at the same time; the nature of the resulting product is not known to date. On the other hand, comparison of the action of slices of spleen with ground spleen, under aerobic as well as anaerobic conditions, shows that slices of spleen under both conditions, and ground spleen under anaerobic conditions, convert cysteic acid into

taurine. Ground spleen under aerobic conditions behaves like the intestinal mucosa, acting simultaneously on the carboxyl group and on the amino group of cysteic acid.

The formation of sulfoxyacetic acid has been shown to be probable by the observations of Schmidt and Clark (105), according to whom ingestion of cysteic acids in dogs, without simultaneous ingestion of cholic acid, results in the appearance of an organic substance in the urine which contains sulfur and amino nitrogen. These observations are in agreement with experiments carried out by Cohen (30) on the ability of cysteic acid to participate in transamination reactions in the muscle according to the following equations:



The sulfoxyacetic acid eventually produced in these reactions could be excreted as such; this would explain the results obtained by Schmidt and Clark.

Medes and Floyd (87) have posed the problem of the stability of sulfoxyacetic acid in the organism, and, particularly, whether this substance could be degraded to sulfate. Their experiments were carried out in the following manner:

A mixture of cysteic acid with oxaloacetic or  $\alpha$ -ketoglutaric acid was maintained for one hour at pH of 7.7 in a 0.07 *M* phosphate buffer solution with transaminase, prepared according to Cohen's technic. Under these conditions transamination amounts to 15 to 18%. To the mixture so obtained and consequently containing a certain amount of sulfoxyacetic acid, they added either ground liver or ground muscle and then maintained the reaction mixture for two hours in presence of oxygen. In each of these experiments the authors were unable to show any appreciable formation of sulfate from sulfoxyacetic acid.

One must thus conclude that the sulfoxyacetic acid eventually formed during the transamination reactions is not a sulfate producer in the organism. Its fate is therefore undetermined.

#### C. DESULFURATION OF CYSTEINE WITH LIBERATION OF HYDROGEN SULFIDE

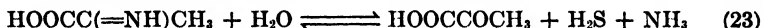
As in the case of homocysteine, cysteine sulfur may be split off the organic molecule in the form of hydrogen sulfide. This desulfuration is

due to the existence of an enzyme, cysteine desulfhydrase, which has been studied independently by Fromageot and collaborators and by Smythe and his co-workers. The principal characteristics of this enzyme, as well as a bibliography concerning it, have been the object of papers by Smythe (112), one of which is contained in a previous volume of this publication (113). It is thus unnecessary to discuss the enzyme in detail here.

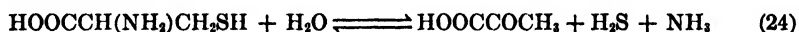
We may recall, however, that the reaction which cysteine undergoes under the influence of the enzyme in question is the following (111):



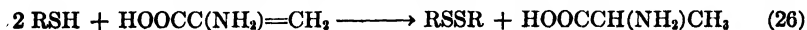
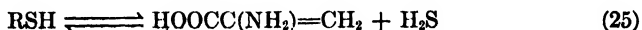
The  $\alpha$ -aminoacrylic acid produced gives rise to the following spontaneous reactions:



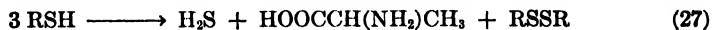
which finally leads to:



Reaction (24) can be observed only with a sufficiently purified enzyme preparation, and by avoiding an excess of cysteine as Smythe did (111). If one uses as the enzyme preparation an aqueous extract of the powdered organ which was obtained by treating ground liver with ether or acetone, one obtains in addition to hydrogen sulfide, no pyruvic acid and ammonia, but alanine and cystine (44,52). This result corresponds to the following equations:



Combining these two equations, one obtains:



These reactions are explained by the presence of an enzyme of yet unknown nature in the enzyme preparation which catalyzes the oxidation-reduction between cysteine and  $\alpha$ -aminoacrylic acid (or iminopyruvic acid).

On the other hand, one can purify the above enzyme preparation by treating it with chloroform (44). Using the enzyme system so obtained, in the presence of a sufficient amount of cysteine (20 mg. per ml. enzyme solution), one notes the formation of ammonia, lactic acid, and cystine, in addition to hydrogen sulfide, according to equation (24) combined with:



to give:



In this fashion, under the more or less direct action of cysteine desulfhydrase, cysteine gives rise to  $\text{C}_3$  molecules, namely pyruvic acid, lactic acid, and alanine. These compounds can easily change one to the other in the tissue. The formation of these molecules establishes the link between the metabolism of cysteine and the carbohydrate metabolism, and explains, among other things, that cysteine must be the generator of "extra glucose" in phlorizinized dogs (31).

The activity of cysteine desulfhydrase is completely inhibited by hydrogen cyanide, hydrazine, phenylhydrazine, hydroxylamine, or semicarbazide in concentrations equal to or less than  $0.001 \text{ M}$ , demonstrating the presence and the importance of the carbonyl group in this enzyme (46,74,82). Furthermore, the activity of the same enzyme is considerably slowed up by a series of substances (in concentrations of  $0.01$  to  $0.1 \text{ M}$ ) which possess an acidic group, or an acidic group and a basic group. This permits the conclusion that in cysteine desulfhydrase groups corresponding to these exist, which may be depicted schematically by  $-\text{N}^+$  and  $-\text{COO}^-$  (46). A comparison of the behavior of this enzyme toward all of the inhibiting substances, indicates that the three active groups are adjacent and placed in the order  $-\text{CO}, -\text{COO}^-, -\text{N}^+$ . According to Fromageot and Grand (46), the union of cysteine and the enzyme brings these three groups into play with the probable transitory formation of a semithioacetal derivative. The dissociation constant of the cysteine desulfhydrase-cysteine complex (Michaelis constant) is  $10^{-2.01}$  at an optimum pH of 7.2 and at  $37^\circ\text{C}$ .; it varies with pH, increasing at either side of pH 7.2.

Among the inhibitors of cysteine desulfhydrase, homocysteine is one of the most interesting. The considerable inhibition displayed by this substance corresponds with the competition which it exercises toward cysteine in the fixation of the carbonyl group of the enzyme. In the presence of  $0.001 \text{ M}$  *dl*-homocysteine, the Michaelis constant has the apparent value of  $10^{-1.76}$ . *d*-Homocysteine possesses a slightly greater inhibitive action than the *l*-derivative (47). Serine, glycolic acid, and probably all inhibitors with acidic and basic group or groups, act in blocking the active  $-\text{COO}^-$  and  $-\text{N}^+$  groups of the enzyme, without markedly interfering with the combination of cysteine and the enzyme by virtue of the carbonyl group.

The fate of the hydrogen sulfide liberated by the action of cysteine desulfhydrase is discussed on page 397.

#### D. CONVERSION OF CYSTEINE TO $\beta$ -THIOLPYRUVIC ACID

Like methionine, cysteine as an  $\alpha$ -amino acid can undergo oxidative deamination, giving rise to  $\beta$ -thiolpyruvic acid. This reaction takes place through the action of the same enzymes, the *d*-amino acid oxidase, and the *l*-amino acid oxidases, which act on methionine and which have been discussed on page 374. No facts are available on the fate of the keto acid in question which apparently does not seem to play an important role in the formation of sulfate or taurine.

### V. Metabolism of Cystine

Like cysteine, cystine is both a primary substance of animal metabolism and an intermediary in the transformations in which methionine or cysteine form the starting materials.

#### A. OXIDATION OF CYSTINE SULFUR *IN VIVO*

The oxidation of cystine sulfur in the animal leads to the formation of sulfate and taurine, among other products.

*The oxidation of l-cystine to sulfate* is a classical fact which is based mainly on the observations of Stearns and Lewis (115) and Virtue and Lewis (138) with rabbits, on those of Hele and Pirie (59) with dogs, and on those of Medes (84) with man. Moreover, as in the case of cysteine, the oxidation to sulfate does not behave differently for *l*- or *d*-derivatives. Hele and Pirie (59) have pointed out that 70% of the sulfur of *l*- and of *dl*-cystine, introduced into dogs by ingestion or injection, is excreted after 48 hours in the form of sulfate, just as in the case of *l*- and *dl*-cysteine. In spite of the frequently observed analogy in behavior between cystine and cysteine in this respect, and, in spite of the ease with which free or combined cystine, particularly in the form of glutathione, is reduced to cysteine in the organism (1,2,36,62,63,81), there are cases in which pronounced differences in behavior between these two amino acids appear in the living animal. According to Medes (84), sulfur oxidation in man, as evidenced by excretion of urinary sulfate, is slower after ingestion of *l*-cystine than after ingestion of the equivalent amount of *l*-cysteine, reduction of cystine to cysteine, when it takes place, being much more rapid than the oxidation to sulfate. One must therefore conclude that oxidation of cystine may take place without previous reduction of the latter substance to cysteine. In particular, the observations on subjects with cystinuria (man or dog) show, as has been pointed out by Brand, Cahill, and Harris (27), the profound differences which can exist between the metabolism of cystine and cysteine.

As in the case of methionine and cysteine, the oxidation of cystine sulfur to sulfate in animals is often considerably slowed up, or even completely inhibited, by blocking of the amino group. This pertains particularly to the observations of Lewis, Updegraff, and McGenty (81) on dibenzoylcystine ingested by rabbits, of Lewis and Root (80) and Virtue and Lewis (138) on phenyluraminocystine, also with rabbits, of Hele and Pirie (59) on dichloroacetylcystine ingested by dogs, and of Jen and Lewis (64) on the betaine of *l*-cystine, injected subcutaneously into rabbits.

Conversely, according to observations of du Vigneaud, Loring, and Craft (133) with rabbits, the sulfur of acetyl and formyl derivatives of *l*-cystine is oxidized just about as in the case of *l*-cystine itself. However, the sulfur of the corresponding derivatives of *d*-cystine is considerably more resistant to oxidation than the sulfur of *d*-cystine.

*The oxidation of cystine sulfur to taurine* is likewise a classical fact since the work of von Bergmann (9) and of Foster, Hooper, and Whipple (40), confirmed by Virtue and Doster-Virtue (134), has demonstrated that simultaneous ingestion of cystine and cholic acid in dogs with bile fistulas produces a considerable increase in the amount of taurocholic acid synthesized in the animals. In these experiments, the starvation nature of the diet to which the animal had been submitted before is of great importance. One must employ dogs, as was done by Virtue and Doster-Virtue, which have been suitably fasted prior to ingestion of the compounds in question.

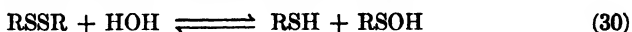
## B. MECHANISMS OF OXIDATION OF CYSTINE SULFUR

### 1. *Prior Reduction to Cysteine*

Several observations demonstrate that cystine, independently of the path of introduction into the organism, is easily reduced to cysteine. According to Lewis, Updegraff, and McGenty (81), over 50% of phenyluraminocystine and dibenzoylcystine (which escape the oxidation to sulfate) introduced into rabbits by ingestion or injection is converted to the corresponding cysteine derivatives. Such a reduction results for the most part from redox reactions in which a number of hydrogen donors present in the tissues may participate. Of particular interest at this point are the sulfhydryl groups of certain enzymes, because their oxidation to disulfide groups, coupled with the reduction of cystine to cysteine, may lead to the inactivation of these enzymes. This, for example, is the case with succinoxidase (4). Among hydrogen donors one should also cite hydrogen sulfide, resulting from the desulfuration reactions discussed above. It is obtained

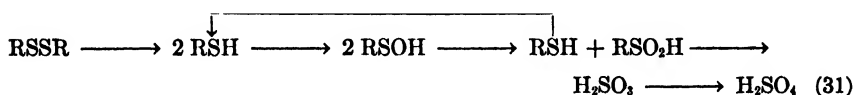
in reaction (13) (page 385) going from right to left. This reaction explains the presence of small amounts of free sulfur, as reported by Smythe (111) in his experiments.

To a lesser extent, the reduction of cystine to cysteine may result from a hydrolysis which is probably not enzymic. This produces a molecule of sulfenic acid and one molecule of cysteine according to:



The possibility of such a hydrolysis has been particularly discussed by Medes and Floyd (86).

Whatever the mechanism of cysteine reduction, it is evident that, if the above reaction takes place, cystine metabolism approaches cysteine metabolism. This is admitted by Pirie (95) in his scheme to illustrate the oxidation of cystine to sulfate by rat liver slices:



## 2. Direct Oxidation of Cystine Sulfur

The hydrolysis reaction discussed above leads to a primary direct oxidation of cystine sulfur, which is converted to cysteinesulfenic acid. We have discussed the fate of this acid on page 386.

In addition, Medes and Floyd (86) have presented evidence that in the liver of rats two enzyme systems exist which bring about the oxidation of cystine. One of them is cysteine oxidase B, which was described on page 388, and which acts likewise on cystine to form cysteic acid; the rate of this oxidation is slightly less than the corresponding oxidation of cysteine. The other is a cysteine oxidase which results in the formation of cystine disulfoxide, RSOSOR. Greenstein and Leuthardt (55) have studied the distribution of cystine oxidase in the different organs of mice. Only liver, kidney, and pancreas, in order of decreasing activity, contain this enzyme. On the other hand, no noticeable activity of cystine oxidase has been revealed in tumors in the same animals.

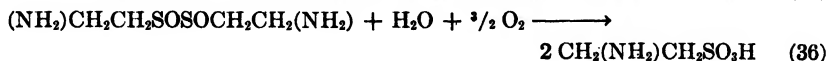
The metabolism of cystine disulfoxide itself has been the object of a number of investigations. According to Medes (84), ingestion of cystine disulfoxide in man is followed by a considerable increase in urinary sulfate. Two theories have been presented to explain the formation of sulfate from cystine disulfoxide. The first one assumes that, prior to oxidation to sulfate, cystine disulfoxide is reduced in the organism to cystine. This theory



is based on experiments by Bennett (7), according to whom cystine disulfoxide can replace cystine in rats fed a cystine-free diet. In these experiments, one molecule of the disulfoxide is equivalent to one molecule of cystine. But Medes points out that since the organism finds sufficient cystine in the diet, there exists the other possibility that cystine disulfoxide can be transformed in a dismutation. This was studied by Lavine (73) and results in the formation of cysteine and cysteinesulfinic acid according to the scheme:



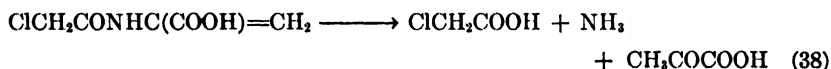
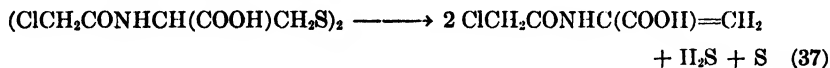
Nevertheless, the sulfate represents only a small portion of the metabolism of cystine sulfoxide. The most important conversion this material undergoes is one which leads to the formation of taurine. Using dogs provided with bile fistulas, and conditioned with a suitable starvation diet, Virtue and Doster-Virtue (135) were able to produce an abundant quantity of taurine in these animals, which was excreted in the form of taurocholic acid, after disulfoxide was injected subcutaneously and cholic acid was introduced by ingestion. These results obtained *in vivo* are in good agreement with the discovery by Medes and Floyd (86) of an enzyme system which is capable of decarboxylating cystine disulfoxide and which is different from cysteic acid decarboxylase. Unfortunately, instability and poor solubility of cystine disulfoxide have deterred Medes and Floyd in the pursuit of their studies of this enzyme. The action of this enzyme, together with the action of an oxidase on cystamine disulfoxide would lead to the formation of taurine:



### C. DESULFURATION OF CYSTINE WITH LIBERATION OF HYDROGEN SULFIDE

Aside from cysteine desulphydrase, which acts only on cysteine in the free state, there exists, according to Greenstein and Leuthardt (56), an exocystine desulphydrase in the liver of rats. This enzyme acts on peptides containing cystine, and possessing an amino group and a free carboxyl group with the cystine at one extremity. The enzyme does not act on any of the following substances: *S*-benzylcysteine, reduced or oxidized glutathione

and diketopiperazines of *l*-cystine and *l*-cysteine. Exocystine desulfhydrase acts more frequently in conjunction with dehydropeptidase. This conjugated action leads to the production of hydrogen sulfide, pyruvic acid, and ammonia. A study of the enzymic mechanism shows that the decomposition of such a peptide containing cystine takes place in three consecutive stages: an enzymic desulfuration by exocystine desulfhydrase, which gives rise to hydrogen sulfide, sulfur, and a dehydropeptide which is unsaturated in the  $\alpha, \beta$ -position; hydrolysis of the dehydropeptide by dehydropeptidase, giving rise to aminoacrylic acid; spontaneous hydrolysis of the latter substance to pyruvic acid and ammonia. A particularly illustrative example of the over-all reactions is furnished by the enzymic decomposition of dichloroacetyl cystine (78):



According to Leuthardt and Greenstein (78), the system exocystine desulfhydrase-dehydropeptidase is the only intracellular enzyme system known to date which is present in most normal tissues but always absent in tumors of the same tissues.

#### D. CONVERSION OF CYSTINE TO THE CORRESPONDING KETO ACID

Cystine undergoes oxidative deamination according to mechanisms analogous to those discussed above for methionine and cysteine. This oxidative deamination leads evidently to the formation of dithiopyruvic acid, the metabolism of which is poorly understood and does not seem to play any important role in animal metabolism.

### VI. Oxidation of Hydrogen Sulfide and Free Sulfur

We have already seen that hydrogen sulfide can originate in animal tissues through the action of intratissue enzymes, from homocysteine, from cysteine, and from polypeptides with exocystine. To this hydrogen sulfide of endogenous origin, one must add such hydrogen sulfide which penetrates the intestinal wall after having originated by bacterial action in the digestive tract. One deals here with a particularly important problem, because of the high toxicity of this substance. Already the older experiments of Haggard (57) have shown the ability of blood and even of plasma to oxidize hydrogen sulfide. This author, in experiments *in vivo*, has

pointed out that it is possible to inject into dogs intravenously amounts of sodium sulfide considerably higher than the lethal dose, provided that this injection is sufficiently slow. This must obviously be due to the ability of tissues to oxidize sulfide. The velocity of oxidation is such that the quantity of sulfide present in the tissues at each instant during the course of injection is always below the lethal dose. This oxidation, according to Denis and Reed (33), leads to the formation of sulfate. The study of the oxidation and the fate of hydrogen sulfide in the organism was recently studied by Dziewiatkowski (39), who used sodium sulfide containing radioactive sulfur,  $^{35}\text{S}$ . In experiments with rats fed 1.66 mg. of sulfur as sodium sulfide, this author obtained the results shown in Table II.

TABLE II  
MARKED SULFUR RECOVERED 24 HOURS AFTER INGESTION.

Tissue	Rat I (215 g.)		Rat II (208 g.)	
	Tissue weight, g.	S, per cent of ingested	Tissue weight, g.	S, per cent of ingested
Sulfur of free urinary sulfate	100*	41.0	100	34.7
Sulfur of total urinary sulfate	100	49.1	100	48.2
Total urinary S	100	78.5	100	51.7
Feces	0.71	4.23	1.28	2.31
Gastrointestinal tract	11.56	6.33	10.61	6.07
Bone	23.43	2.40	22.67	2.15
Heart	0.47	0.017	0.59	0.014
Kidneys	1.76	0.15	1.76	0.14
Spleen	0.31	0.03	0.31	0.02
Gonads	4.52	0.09	3.90	0.11
Lungs	0.82	0.05	0.94	
Blood	12.80	0.45	12.36	0.53
Liver	5.66	0.13	8.76	0.33
Brain	1.80	0.02	1.66	0.02
Skin	38.70	1.47	37.44	0.43
Muscle	97.61	0.85	94.43	0.64
Hair	4.17	0.005	4.72	0.016
<i>Total</i>	203.61	94.722	200.15	64.480

\* Urine diluted to 100 ml. The 100 ml. obtained was assumed to weigh 100 g.

These results show unquestionably the ability of the organism to oxidize the sulfur of sulfide to sulfate. In addition they indicate the possibility that hydrogen sulfide re-enters organic linkage, probably in tissue proteins. The possibility is in agreement with the reversible action of cysteine desulfhydrase, shown by Smythe and Halliday (114). These authors mixed cysteine containing ordinary sulfur, sodium sulfide contain-

ing marked sulfur ( $^{35}\text{S}$ ), and ground liver, and stated that an exchange of the ordinary sulfur of cysteine with the labeled sulfur takes place.

The mechanism of the oxidation of sulfide sulfur to sulfate is probably much more complex than a simple fixation of oxygen. Fromageot and Royer (51) have presented the hypothesis that the oxidation of hydrogen sulfide to sulfate involves formation of thiosulfate as intermediary. They base this hypothesis on the constant presence of thiosulfate in the urine of higher animals, and also on the ease with which hydrogen sulfide is oxidized to thiosulfate by inorganic catalysts in the presence of air (106).

We have seen (page 394) that hydrogen sulfide can undergo another type of oxidation in tissues: acting as hydrogen donor, it gives rise at the same time to free sulfur. Numerous hydrogen acceptors, particularly the disulfide groups in proteins, participate in this reaction. [Bersin (13) has listed the enzymes activated by the reduction of their disulfide groups to sulfhydryl groups by the action of hydrogen sulfide.] The sulfur thus formed is easily reconverted to the sulfide state by the action of various reducing agents in the tissues. It can also be oxidized to sulfate. The mechanism of the last-mentioned oxidation is yet unknown; it possibly implies a previous reduction to hydrogen sulfide. Experiments of Greengard and Woolley (54), however, demonstrate that colloidal sulfur, ingested by man in a dose of 500 to 750 mg. per day, is completely absorbed, oxidized, and excreted in the urine as sulfate. These phenomena are very rapid, since the increase in urinary sulfate appears as soon as two hours after ingestion. This rapidity seems to indicate that the oxidation of sulfur takes place without prior reduction to sulfide.

## VII. Metabolism of Thiosulfate

The formation of thiosulfate in animals has been mentioned repeatedly. Urinary thiosulfate of higher animals has long been the subject of numerous investigations, of which the most important were recalled by Fromageot and Royer (51), who have studied the origin of thiosulfate in experiments with cats, guinea pigs, rabbits, and rats. They could demonstrate that the amount of thiosulfate excreted in the urine is always considerably larger in animals—carnivorous or herbivorous—if they are previously kept on a starvation diet than if they are fed normally. This phenomenon is completely reversible.

These results show that urinary thiosulfate comes from the animal metabolism proper, and not, as had been believed for some time, from bacterial action in the digestive tract. It thus represents an intermediary

product in the oxidation of organic sulfur in animal metabolism, by consumption of endogenous proteins in the starvation period, or from the proteins afforded in the diet.

To the thiosulfate in the urine, thiocyanate should be added, which according to Lang (72) arises from the following reaction between thiosulfate and hydrogen cyanide:



This reaction is catalyzed by an enzyme, rhodanese, which is present in different tissues.

The mechanism of thiosulfate formation in animals is still obscure. Three theories can actually be proposed for this problem: according to one by Medes and Floyd (86), thiosulfate would be formed from sulfoxylic acid; according to one by Fromageot and Royer (51), it would be formed by oxidation of hydrogen sulfide; and finally one may think thiosulfate would result from the reaction between free sulfur and sulfite.

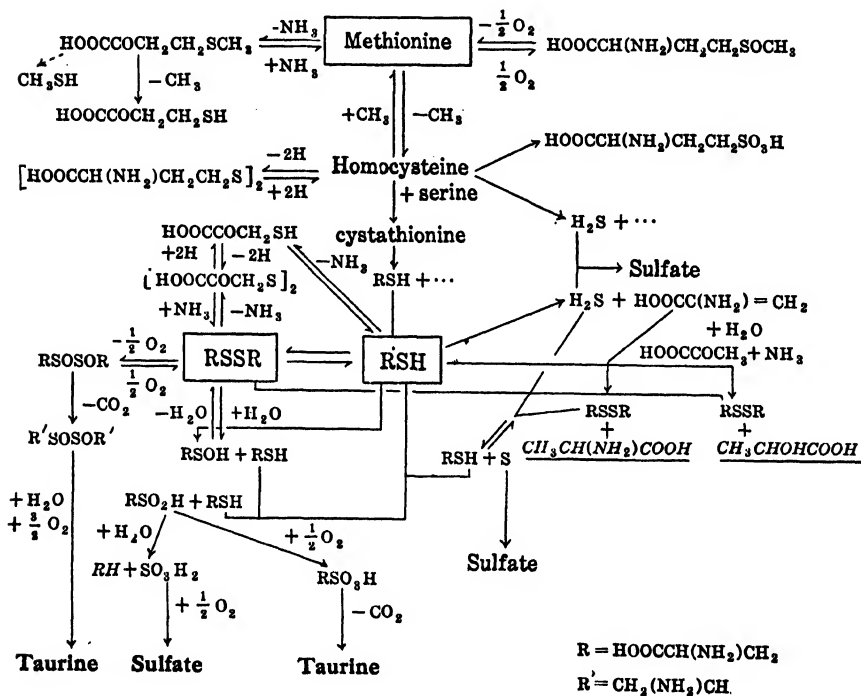
Whatever the mode of formation, the role of thiosulfate as an intermediary compound in the oxidation of organic sulfur to sulfate is definitely demonstrated by the ability of higher animals to oxidize thiosulfate to sulfate *in vivo* (92,122,150). This oxidation was also demonstrated by Pirie (96) in his experiments with tissue slices.

### VIII. Over-all Picture of the Oxidation of Organic Sulfur in Animals

A summary of the preceding considerations is shown in the accompanying scheme, in which the substances in the boxes represent primary materials, the substances in heavy type represent the corresponding forms resulting from the oxidation of organic sulfur, and the italicized substances denote compounds which, after having lost their sulfur, continue to participate in general metabolism.

This schematic representation deserves some comments. Several possibilities can be seen on examining the relationships which exist between sulfur oxidation and the linkage of sulfur with organic molecules of which it forms a part. On the one hand, sulfur can be detached from homocysteine, cysteine, or cystine *before any oxidation*. This fission, which takes place under the action of various desulfhydrases, gives rise to hydrogen sulfide which is then oxidized to sulfate in poorly understood stages. On the other hand, sulfur may be *partially oxidized* and thus remain bound to the organic molecule. In the more important case of cysteine, the oxidation goes to the

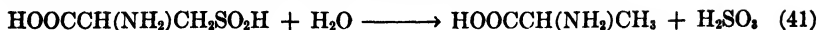
stage of sulfinic acid. At that stage, the sulfur is separated from the organic residue by the action of desulfurase and the sulfurous acid so liberated is oxidized to sulfate. Finally, the sulfur can remain entirely linked with the organic molecule, being *oxidized completely*, and leading to the formation of cysteic acid. The sulfur is now not detached from the organic molecule.



The latter is decarboxylated by the action of cysteic decarboxylase and the oxidized sulfur is excreted in the form of taurine. At that time it is of interest to compare the action of desulfenicase with the action of decarboxylase. There seems to exist a certain analogy between these enzymes, just as there is an analogy between the sulfinic and carboxyl groups on which they act. But the experiments carried out to date do not show whether the desulfenicase acts by splitting off sulfur dioxide from cysteine sulfinic acid according to the equation:



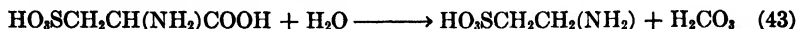
or whether it eliminates the sulfurous acid according to:



Likewise, one cannot say whether the decarboxylases, or at least certain ones of them, eliminate carbon dioxide according to the equation:



or the carboxyl group according to:



Here is a problem in comparative enzymology which should be interesting to solve with the aid of marked oxygen.

In regard to the paths leading from cysteine and cystine and terminating in sulfate, the fact that the oxidation of cystine to sulfate is much slower than the oxidation of cysteine, *in vivo* as well as *in vitro* with enzyme preparations, leads to the conclusion that the hydrolysis of cystine into sulfenic acid and cysteine is much slower, and consequently plays a much less important role in metabolism than does the oxidation of cysteine to sulfenic acid.

Because of the order in which nitrogen and sulfur are eliminated after ingestion of cystine or cysteine, and of the suppression of sulfur oxidation by blocking the amino group of cystine or cysteine, some authors (80,105) have thought that the oxidation of sulfur to sulfate implied a previous deamination of cystine or cysteine. It is—on the contrary—a well-established fact that, in the majority of cases, the oxidation of sulfur or its fission from the organic molecule takes place without any previous deamination. This is evident in the majority of paths of degradation shown in the scheme. Inhibition of sulfur oxidation by blocking of the amino group is easily explained by the fact that such a blockage suppresses the affinity which the sulfur-containing organic molecule has for the enzyme which brings about its oxidation. Concerning the fact that, in the metabolism of cystine or cysteine, nitrogen appears to be eliminated before sulfur, many other reasons than previous deamination of the organic molecule can serve as explanation.

## IX. Oxidation of Sulfur of Various Substances

Numerous substances containing sulfur which normally do not participate in animal metabolism have been studied with respect to their ability to be oxidized in the organism. This oxidation is most often characterized by the formation of sulfate which is excreted in urine. Without discussing

the details of these studies, nor the toxicity and physiological effects of these substances, we have summarized the results of some of them with respect to sulfur oxidation in Table III. It thus appears that the sulfur of sulphydryl

TABLE III  
SULFUR OXIDATION IN VARIOUS TEST ANIMALS

Substance	Animal and path of introduction	Formation of urinary sulfur	Ref. No.
Ethyl mercaptan, $C_2H_5SH$	Dog, ingestion	0	110
Thioglycolic acid, $CH_2(SH)COOH$	Rabbit, ingestion or subcutaneous injection	++	60
Thiolactic acid, $CH_3CH(SH)COOH$	Rabbit, ingestion or subcutaneous injection	++	60
Thiophenol, $C_6H_5SH$	Rabbit, ingestion or subcutaneous injection	0	61
Thiocresol, $CH_3C_6H_4SH$	Rabbit, ingestion or subcutaneous injection	0	61
Thiouamil, $HN-C-SH$ $OC-C=NH_2$	Dog, subcutaneous injection	+	43
$\gamma$ -Thiopseudouric acid, $HN-CO$ $HN-CO$ $OC-CH-NH-C-SH$ $HN-CO$ $NH$	Dog, subcutaneous injection	+	43
Thiourea, $SC(NH_2)_2$	Rabbit, subcutaneous injection	0	103
Ethyl thiocarbamate, $NH_2CSOC_2H_5$	Dog, ingestion	++	109
Thiodiglycolic acid, $S(CH_2COOH)_2$	Rabbit, ingestion or subcutaneous injection	0	60
2-Thiohydantoin, $NH-CO$ $SC-NH-CH_2$	Rabbit, ingestion or subcutaneous injection	0	79
2-Thio-4-methylhydantoin	Rabbit, ingestion or subcutaneous injection	0	79
2-Thiohydantoin-4-acetic acid	Rabbit, ingestion or subcutaneous injection	0	79
Thiourethan, $NH_2COSC_2H_5$	Dog, ingestion	0	109
Isothionic acid, $CH_2OHCH_2SO_3H$	Dog, ingestion	0	105

groups attached to an aliphatic chain or to a heterocyclic ring is sometimes oxidized to sulfate, although the sulfur of ethyl mercaptan is not oxidized.



Sulfur of sulfhydryl groups directly attached to the benzene ring are remarkably resistant. And, finally, the sulfur of thiourea and its derivatives (thiohydantoins) likewise escapes all oxidation.

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# INTERRELATIONS IN MICROORGANISMS between Growth and the Metabolism of Vitamin-like Substances

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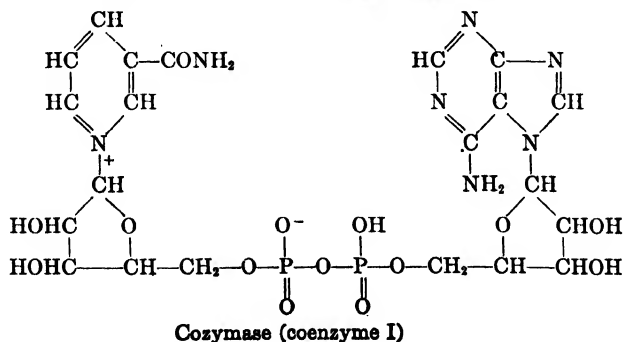
## I. Introduction

Most microorganisms can bring about chemical changes in substances such as glucose, lactate, or glycerol with velocities of the order of several micromoles per milligram dry weight of organism per hour; it is proposed to refer to these as reactions of micromolar order. Corresponding to such changes is an equal or greater turnover of coenzymes and allied substances which play catalytic parts in the reactions. In addition, the coenzymes are formed or assimilated, excreted or inactivated. Relatively little atten-

tion has been paid to the nature or speed of this latter category of change although its importance is obvious from its indispensable part in microbial growth. It is quantitatively much more evident in microorganisms, and especially in bacteria, than in animal or plant tissues as they are ordinarily studied, because of the rapid growth of bacteria. In microbial cultures capable of doubling in population each 15 or 30 minutes, the microbial substance itself is a major metabolic product. Thus streptococcal glycolysis is among the rapid bacterial processes, and can result in the formation of 15 to 30 micromoles ( $\mu\text{M}$ ) of lactic acid per milligram dry weight of streptococci per hour. This implies production of a quantity of the acid about equal in weight to that of the organisms, in half an hour. In this time the organisms are capable also of producing more than their own weight of new streptococci, including a quantity of coenzymes more than that originally present. In a population of stable coenzyme content there must thus be a balance between rate of growth of population, dependent on reactions such as glycolysis, and the rate of synthesis or assimilation of coenzymes. The means by which such processes may be connected have been considered below, by reviewing the behavior of nicotinic acid derivatives and of pantothenic acid, both of which exist in microorganisms in quantities of the order of millimicromoles ( $\text{m}\mu\text{M}$ ) per milligram dry weight. Several reactions with velocities of the order of millimicromoles per milligram dry weight per hour (briefly, of  $\text{m}\mu\text{M}$  order) have been discovered in such substances, and, as will be seen, these changes show a certain linkage with reactions of the micromolar order.

## II. Nicotinic Acid and Its Derivatives

Observation of the inactivation of cozymase by yeast preparations was contemporary with the discovery, at the beginning of the present century,



(see 36,37) of cozymase itself, but the process was not studied in detail until about the time when cozymase was being purified and its structure was being determined. This followed the elucidation, in 1936, of the nature of coenzyme II, which contains one more phosphoric acid group than cozymase (see 117). Association of the two coenzymes with bacterial growth and metabolism was first reported by Lwoff and Lwoff in 1937, in two papers (60,61) which form excellent examples of the subject of the present review, and which are considered first below.

#### A. *HEMOPHILUS PARAINFLUENZAE* AND NICOTINIC ACID DERIVATIVES

##### 1. *Stimulation of Metabolism by Coenzymes I and II*

Several observations on the behavior of influenza bacilli toward "V factor," before chemical characterization of the factor, had suggested certain connections with the metabolism of the bacteria (see 106) though only in very general terms. More specific was Pittman's (95) observation of the lesser need by *H. influenzae* for V factor when culture fluids (containing excess of other growth essentials) were given good contact with air.

Immediately following their demonstration that V factor could be replaced by reasonably pure coenzymes I and II, Lwoff and Lwoff (61) examined the physiological relationship between *H. parainfluenzae* and these substances. Their working hypothesis was that the coenzymes were used as such by the bacteria. The effects of the substances on the ability of suspensions of *H. parainfluenzae* to transfer hydrogen from a normal constituent of their growth media—namely, glucose—to methylene blue, was accordingly examined. Support for the hypothesis was immediately obtained. Organisms which had been grown in the presence of excess V factor were found to reduce methylene blue much more quickly than those grown with limiting quantities of the substance. Moreover, the deficiency of the latter organisms was specifically related to the lowered quantity of V factor, for it could be made good even in nonproliferating organisms by the addition of coenzymes. Their effect was manifest after a short incubation period of about 2.5 minutes. The coenzymes had no effect on hydrogen transport by organisms grown with excess of V factor, and, from the response of deficient organisms to added coenzymes, the extent to which their dehydrogenation was limited by coenzymes could be calculated and was expressed as a per cent saturation of the organisms with respect to the coenzymes. By growing for 26 hours with little V factor, cultures were obtained only 5–15% saturated with respect to the coenzymes.



The response to the coenzymes which the authors observed was not confined to the convenient but relatively artificial circumstance of hydrogen transfer to methylene blue; a stimulation of respiration and glycolysis occurred to about the same extent as stimulation of methylene blue reduction. The increase in hydrogen transport by limiting quantities of coenzyme was roughly proportional to the quantities of the coenzyme that were added, and from such observations it was possible to estimate approximately the turnover number of the coenzyme. This indicated that, at 38°C., one mole of coenzyme reduced per second about one mole of methylene blue. The value is low in comparison with turnover numbers found for the coenzymes in separated enzyme systems (see 34) and this was attributed (61) to lack of complete absorption or utilization of the substance by *H. parainfluenzae*. It is compared below with related values observed in other microorganisms.

## 2. Coenzyme Inactivation during Dehydrogenation

A prominent feature of the Lwoffs' studies was the simple manner in which further reactions of the coenzymes were connected with their functioning. In doing this, much depended on the different properties of coenzymes I and II, which in the observations described in the previous paragraphs had not behaved differently.

The effects of added coenzymes on dehydrogenation were examined during and after the growth of cultures in glucose-containing media. The organisms were found initially to be saturated with respect to coenzymes, and to remain so during the greater part of the culture's growth. After this their activity fell steadily, but could be restored within a few seconds by small quantities of cozymase; the fall was therefore due only to lack of cozymase, and was found to correspond to an inactivation of 30% per hour of the coenzyme present in the cell at a given time. If the initial cell content of cozymase approximated values found for nicotinic acid derivatives in several other bacterial species (2  $\mu$ M per mg. dry weight; see page 424) this would imply an initial rate of coenzyme inactivation of 0.6  $\mu$ M per mg. dry weight per hour.

The fall in coenzyme activity was connected with the presence of glucose in the growth media, for organisms grown in the same manner but in a peptone medium without added glucose were able to transfer hydrogen rapidly from glucose to methylene blue, at a time when cultures grown with glucose could do so only slowly. It was concluded that cozymase was being "used up" while "and probably because" it functioned in hydrogen

transport. This was supported by examination of the stability of the coenzymes during other dehydrogenations, both in organisms grown in the normal manner with glucose, and in others grown with different substrates in place of glucose. In all cases the bacteria were grown with limited amounts of cozymase. Such experiments comprised two parts: (1) growth on various substrates, which was found to influence the behavior of the organisms during (2) exposure of the harvested and washed organisms to a variety of other substrates in the presence of methylene blue, with and without added coenzymes. Organisms grown from glucose or hexose monophosphate could not immediately dehydrogenate pyruvate, fumarate, ethanol, glucose, or hexose monophosphate. The ability to dehydrogenate these substances was restored by added coenzymes; coenzyme activity had been lost during growth. If, however, pyruvate or fumarate was the substrate for growth, the resulting organisms were able to dehydrogenate glucose and hexose monophosphate at rapid rates which were uninfluenced by added cozymase. Such growth had not involved usage of coenzymes concerned with glucose. These organisms were, however, relatively slow in dehydrogenating pyruvate or fumarate; the rates of these reactions were increased by added coenzymes.

Thus deficiencies in each type of organism were made good by either coenzyme. In inducing deficiencies, glucose and hexose monophosphate behaved similarly, but differently from pyruvate, fumarate, malate, ethanol, some amino acids, and the peptone of the basal medium.

### 3. Assimilation of the Coenzymes

In interpreting the preceding data a special hypothesis was made (61) with respect to the assimilation of the two coenzymes. When dehydrogenases requiring coenzyme I or II have been separated from organisms, including yeasts or bacteria, they have usually reacted specifically with one or the other of the coenzymes. Thus the hexosemonophosphate dehydrogenase of yeast (86) required coenzyme II, while alcohol dehydrogenase from yeast reacted with coenzyme I (87). The specificity of intact cells of *H. parainfluenzae* toward added coenzymes was not exhibited until the coenzymes were assimilated. Cells could be prepared which were deficient in coenzymes in two different ways, and it was suggested (61) that the difference between the two types of cells lay in their being deficient in either one or the other of the two coenzymes.

Knowing that the hexose monophosphate system of other organisms required coenzyme II, it was assumed that the cells unable to dehydrogen-

ate hexose monophosphate (and also glucose) were deficient in coenzyme II. From this it followed that the other type of cell, unable to dehydrogenate pyruvate, fumarate, malate, ethanol, asparagine, and valine, was deficient in coenzyme I. According to the Lwoffs' first hypothesis, these deficiencies in the coenzymes were induced as a result of the reactions in which the coenzymes took part; the deficiencies should therefore indicate the coenzyme requirements of the different dehydrogenases of *H. parainfluenzae*, but the necessary observations with separated systems do not appear to have been carried out. Numerical comparison may be made between the rates of glucose dehydrogenation and of the associated coenzyme inactivation, on the following basis (see page 420): 30% of the organisms' coenzyme is inactivated per hour, while the turnover number shows each mole to transport one mole of hydrogen per second. The dehydrogenation is thus about twelve thousand times faster than the inactivation.

If this indirect evidence for the specificity of the dehydrogenase systems of *H. parainfluenzae* with respect to coenzymes is accepted, it follows that the intact cells are capable both of converting added coenzyme I to II and II to I. When, however, the coenzymes form part of the cells, such changes appear to be prevented. This presumably reflects a type of cell organization which is illustrated also by the rapid inactivation of both coenzymes by many tissues and organisms after their disintegration.

These hypotheses should be reasonably accessible to experimental investigation. In particular, it would be valuable to measure the contents of coenzymes I and II, in variously treated cells, by methods not involving reactions of the coenzymes in those cells; for example, in the systems described on page 424, after extraction.

#### 4. End Products of Coenzyme Metabolism

Having connected the inactivation of coenzymes I and II with their functioning in hydrogen transport, Lwoff and Lwoff (61) suggested that the inactive product might be an "over reduced" or "wrongly reduced" coenzyme, such as can be produced from them chemically. The product has not been characterized, though it would appear easy to determine whether the nicotinamide nucleus remained intact. As nicotinamide nucleoside can function as V factor, the product is presumably not the nucleoside. More extensive investigations have been made of the products of cozymase inactivation in yeasts; the Lwoff's hypothesis is discussed below in terms of these.

### 5. Effects of Nicotinamide Derivatives on Growth of Influenza Bacilli

Nicotinamide riboside is the simplest substance known to function as V factor (33). In this respect the growth response of the organisms has been more fully investigated than their metabolic response. Supposing with Lwoff that the V factor functions as a coenzyme, the action of the riboside implies that growing cells are capable of converting it to coenzymes I or II; a conversion in resting cells such as might be shown by a metabolic response does not appear to have been observed.

Comparison of the activities of different nicotinamide derivatives in supporting growth of *H. influenzae* and *H. parainfluenzae* is not straightforward. The substance found capable (33) of promoting growth of most strains in lowest concentrations was nicotinamide riboside, of which  $0.5 \times 10^{-9} M$  solutions gave just observable growth;  $2 \times 10^{-9} M$  was required of cozymase or its dihydro compound, and a slightly higher concentration of coenzyme II or of "desaminocozymase" (see 105). When, however, the minimum concentrations giving any growth above about 10% of the maximum attainable were considered, nicotinamide riboside or coenzyme II was much less effective than cozymase, its dihydro compound, or desamino derivative. Thus the quantities needed for half-maximum growth with a strain studied in detail were: cozymase,  $2 \times 10^{-8} M$ ; desaminocozymase,  $3 \times 10^{-8} M$ ; nicotinamide riboside or coenzyme II,  $10^{-7} M$ . One strain, when freshly isolated, needed much more of the riboside, but not of coenzyme II, than the majority of strains; this presumably reflected a relative inability on the part of the organism to convert the riboside to a coenzyme. Gingrich and Schlenk (33) interpret other results in a similar manner, suggesting, for instance, that coenzyme II may be converted to the riboside before it is utilized by the organism. The findings referred to above, with the more exacting strain, do not support this suggestion. An alternative explanation of the lesser activity of coenzyme II may be the inactivation which Lwoff found to accompany the organisms' reaction with glucose. Glucose was employed in the growth experiments quoted (33) and observations on the total substances of V factor activity in media and cells before and after growth, were not made. That inactivation of coenzymes during growth can be a factor conditioning the amount needed for growth, is further suggested by a result with a strain of *H. parainfluenzae* which was unusually slow in growth and which—as would be expected if inactivation continued during this slow growth—required more cozymase than usual for its growth. This may be compared with the behavior of streptococci toward arginine. Their requirements for this

substance in growth increase with increase in their arginine dihydrolase activity (31).

## B. COLIFORM ORGANISMS AND NICOTINIC ACID DERIVATIVES

### 1. Stimulation of Respiration and Dehydrogenation

The studies initiated with influenza bacilli have been extended to certain coliform organisms which were later shown to need nicotinic acid or its derivatives, preformed, for their growth. Washed dysentery bacilli grown on a mixture of amino acids, inorganic salts, glucose, and defined quantities of nicotinamide were capable of reducing methylene blue in the presence of glucose (17). The minimum initial concentration of nicotinamide in growth, necessary for optimal speed of reduction by the prepared organisms, was about  $2 \times 10^{-7} M$ . When grown with about  $3 \times 10^{-8} M$  nicotinamide, the speed of reduction was about one-fourth that with nicotinamide-rich organisms, and this deficiency was made good by adding to the washed suspensions either nicotinic acid, its amide, or cozymase. Moreover, the first two compounds acted very promptly in dehydrogenation of glucose; their effect was equal to or greater than that of cozymase in experiments lasting only about three minutes.

A Sonne type of *Shigella paradysenteriae*, prepared in the same manner, was also examined with respect to oxygen uptake, while using glucose as substrate, and by manometric methods (102). In experiments lasting about two hours relative deficiencies much greater than those reported above were shown in the organisms grown from small quantities of nicotinamide; a twentyfold increase in oxygen uptake could be induced by addition of nicotinamide to make a final concentration of about  $3 \times 10^{-6} M$ .

The oxygen uptake per unit time associated with a given quantity of nicotinamide may be calculated from data which Saunders, Dorfman, and Koser (102) record with limiting concentrations of nicotinamide using *S. paradysenteriae*. With concentrations between  $3 \times 10^{-7}$  and  $2 \times 10^{-6} M$ ,  $1 \mu M$  was associated with an oxygen uptake (glucose as substrate) of about  $2500 \mu M$  in 2 hours; this is equivalent to a turnover at an unspecified temperature of just less than one mole of hydrogen per mole nicotinamide per second (see page 412).

When examined by following oxygen uptake the effects of different derivatives of nicotinic acid were not straightforward. Though nicotinamide was more active than nicotinic acid, cozymase was less active than nicotinamide. This observation, initially based on a comparison of nicotinamide with a specimen of cozymase estimated to be 40% pure, was confirmed by showing the activity of such a preparation, and also one of coenzyme II, to be increased on hydrolysis by autoclaving with 0.1 *N* sulfuric acid. Conclusions drawn from these findings are discussed below.

An analogous observation was made in the course of examining the effects of similar preparations on the respiration of *Proteus vulgaris* (102).

The oxidation of glucose by cells of this organism, grown with suboptimal quantities of nicotinamide, was again defective and could be increased three to five times by added nicotinic acid or several of its derivatives. Nicotinic acid, the amide, coenzyme I, and coenzyme II were all capable of giving optimal stimulation when added in excess, but when limiting quantities were used, coenzyme II again had less activity than the products of its hydrolysis. Response to nicotinic acid derivatives was, however, markedly different when oxidation of lactate or glutamate (instead of glucose) was studied with the same organisms, again grown with glucose. In these instances, only coenzyme I was capable of stimulating oxygen uptake; the products of its acid hydrolysis, and even coenzyme II, were incapable of affecting oxidation.

## 2. Interpretation of Coenzyme Activity

Explanations which have been offered of the foregoing results require assessing. Saunders, Dorfman, and Koser (102) are probably correct in their conclusion that the findings as a whole cannot be explained in terms of differential permeability. Some, however, may be. In the presence of glucose, nicotinamide may diffuse into *S. paradysenteriae* or *P. vulgaris* and be converted to a coenzyme more rapidly than the coenzyme itself, of markedly different polar properties, could enter the cells. A mechanism alternative to this is considered below. It appears less feasible that, in the presence of lactate, nicotinamide and coenzyme II should be incapable of entering cells while coenzyme I could do so. Here the explanation most likely, in view of findings with yeast (see page 425), would be in terms of a limitation imposed on coenzyme interconversion or synthesis by replacement of glucose by lactate. In yeasts glucose not only requires the coenzyme for its breakdown but glucose also conditions the inactivation and resynthesis of the coenzyme from substances which include nicotinamide. Morel's opinion—that to suppose such a limitation would imply the organisms had been damaged in handling—is not valid, for her observation (see 28) that *P. vulgaris* can produce coenzymes from lactate as carbon source refers to the behavior of the organisms during their growth, while the metabolic experiments were with resting cells. Her skepticism regarding the suggestion (102) that nicotinamide might function as a hemochromogen is justified, but emphasizes the necessity for investigation of the coenzyme content of these cells, for example, by methods analogous to those applied to yeasts (58).

A further point indicates the need to follow coenzyme synthesis in the

bacteria themselves. This arises from the observation that a further limitation has been imposed on the cells of *P. vulgaris* during the experiments of Saunders, Dorfman, and Koser (102), by growing the bacteria in glucose prior to their metabolism of lactate. Even in the presence of coenzyme I the rate of oxidation of lactate by the bacteria was only one-fifth that of their oxidation of glucose. The adaptation to glucose might well extend to the metabolism of the relevant coenzymes.

### 3. *Inhibition of the Actions of Nicotinic Acid Derivatives*

Exposure of nicotinamide-deficient dysentery bacilli to sulfapyridine largely prevented nicotinamide from having its usual action in stimulating the oxidation of glucose by the organisms (18). Sulfathiazole, but not sulfanilamide, behaved similarly. The present action was thus distinct from that chemotherapeutically major action of the sulfonamide drugs as a group, which is antagonized by *p*-aminobenzoic acid (13). This was confirmed by observation of an equal or greater metabolic inhibition by an acetylsulfapyridine which was ineffective in therapy, and by the inability of *p*-aminobenzoic acid to prevent the effects of sulfathiazole on nicotinamide action. Oxidation of lactate and acid formation from glucose were similarly affected by sulfapyridine; the stimulation given by cozymase was also inhibited.

The magnitude of the inhibition of oxygen uptake by dysentery bacilli, with glucose as substrate, was measured in the presence of varying quantities of nicotinamide and sulfapyridine, with results consistent with the existence of competition between the two substances for an active center of the organisms. Values for equilibrium constants of the hypothetical reaction were reasonably independent of the concentrations of nicotinamide and sulfapyridine. Consistent also with this type of action was the finding that the action of the inhibitors was reversible, and that when they were washed from the cells nicotinamide had its normal action. As the stimulation in respiration given by cozymase itself was inhibited by sulfapyridine, Dorfman and Koser (13) concluded the action of the inhibitor to be on the functioning of coenzymes rather than on their synthesis. For appreciable (85%) inhibition of the action of either nicotinic acid or cozymase, they must, however, be added after the inhibitor; if added before, inhibition is only some 10%. The inhibition was considered (13) to be associated with the structural similarity between the pyridine or thiazole part of the molecules of the drugs and the coenzymes. Glucose dehydrogenase from beef

liver has been found to be inhibited competitively by pyridine-3-sulfonic acid (21) and a similar explanation has been given.

#### 4. Inactivation of Nicotinic Acid Derivatives by *Proteus vulgaris*

Morel (81,82), in Lwoff's laboratory, applied to *P. vulgaris* the methods by which metabolic changes in nicotinic acid derivatives by *H. parainfluenzae* were shown to be connected with their functioning. The application was valuable in referring to an organism of different type and of greater synthetic abilities than the influenza bacilli.

The initial observations were of the dehydrogenating abilities (by methylene blue reduction) of aliquots of whole cultures during their growth on limited quantities of nicotinic acid, in media containing ammonium salts and glucose. Dehydrogenation at first increased, with growth of the bacteria; but later, as the bacterial growth began to be retarded by lack of nicotinic acid, dehydrogenation decreased. The decrease occurred before bacterial growth ceased, and continued after the growth became maximal, although the optical density did not decrease. The fall in rate of reduction indicated the system as a whole to be losing its activity at the rate of about 15% per hour. Morel's data are inadequate for calculation of a metabolic quotient, but supposing her strain of *P. vulgaris* to contain initially the quantity of nicotinic acid derivatives found by Thompson (114) in several organisms, including *P. vulgaris* (namely, about 2  $\mu$ M per mg.; for computation see page 424) the initial rate of inactivation would be 0.3  $\mu$ M per mg. dry weight per hour.

This loss in activity did not occur if cultures were grown with excess nicotinic acid. Also the fall in activity of cultures deficient in nicotinic acid was to a large extent made good by additions of nicotinic acid during the dehydrogenation experiments. Such additions did not affect the reducing powers of suspensions of organisms grown with excess of the acid. The deficient reducing power of cultures limited in nicotinic acid was thus fairly certainly due to lack of available nicotinic acid, and the development of this deficiency indicated some change in nicotinic acid or its derivatives. Morel refers to this change as "usage" (*usure*) of the compounds. It is distinct from a type of inactivation of dehydrogenase systems which occurred during later periods of growth, and which did not respond to added nicotinamide.

A different experimental arrangement permitted the speed and magnitude of response to added nicotinic acid to be more easily observed. Oxygen uptake was determined manometrically with glucose as substrate; again, deficiencies were observed in



cultures grown from small quantities of nicotinamide, and addition of nicotinic acid led to very large increases in respiration. These appeared to commence almost as soon as the acid was added, but required over an hour to reach maximum values. If the quantities of added nicotinamide (82) were small (about 0.1 to 0.2  $\mu\text{M}$  with a quantity of organisms absorbing about 2  $\mu\text{M}$  oxygen per hour), the increase in respiration they caused was again followed by a decrease as the nicotinic acid was inactivated. From the initial increase caused by limited quantities of nicotinic acid, its turnover number could be calculated. In this way 1 mole of nicotinamide was computed to condition the transfer of 2.4 moles oxygen per second. This is equivalent to 4.8 moles hydrogen per second and is thus about 120,000 times more rapid than the inactivation of cozymase with which it is associated (81,82).

### 5. Inactivation of Nicotinic Acid, Associated with Different Substrates

As with *H. parainfluenzae*, an interesting specificity was observed in the behavior of *P. vulgaris* toward nicotinamide derivatives in the presence of substrates other than glucose. The organisms prepared by growth in glucose reacted with pyruvate and methylene blue at only about one-tenth the rate at which they reacted with glucose; but by repeated subculture in solutions containing pyruvate as carbon source, a trained strain was obtained which reacted with pyruvate rather more quickly than with glucose. The rate of reaction with glucose changed little during the adaptation.

When the rate of reduction of methylene blue with pyruvate as substrate was followed during the growth of cultures of the trained organism in limited quantities of nicotinic acid, results were obtained which were analogous to those obtained with the normal strain while employing glucose as substrate. While growth was slowing, or had ceased, the dehydrogenating ability of cultures fell, but could be restored by nicotinic acid, added later. A subsequent phase, in which loss of dehydrogenating power was due to other changes, again followed. The rate of inactivation corresponded to a "usage" of nicotinic acid of about 7% per hour at 37°C.

The reactions of such organisms to the two substrates were sharply differentiated. Organisms deficient in nicotinic acid were grown and examined as washed suspensions prepared from a culture at different times after its phase of maximum growth rate. During this time, while the ability of the organisms to react with pyruvate was falling, their ability to react with glucose remained unchanged. Added nicotinamide restored, partly or completely, reducing activity in the presence of pyruvate without affecting the reaction to glucose. Morel's (82) interpretation of these effects are similar to those discussed on page 413 for *H. influenzae*, namely, that metabolism of pyruvate by *P. vulgaris*, also, involves only coenzyme I. Stored coenzyme II, not undergoing reaction, is not depleted and remains

fully capable of dehydrogenating glucose, but cannot be converted by the organism to coenzyme I. Here again, such conclusions, though reasonable, lack direct confirmation. Usage of added coenzymes by *P. vulgaris* does not appear to have been followed.

#### 6. Nicotinic Acid and Its Derivatives in Growth of Coliform Organisms

**Comparative Activities of Different Compounds.** Coenzymes I and II are less active in promoting growth of *P. vulgaris* in a medium of glucose and inorganic salts than is nicotinamide (82). Acid and alkaline hydrolysis of the coenzymes increased their growth-promoting activities. Explanation of this may follow the suggestions made above in relation to the metabolic effects of the same compounds. In addition, Morel (82) has considered the possibility that the less active coenzymes might be inactivated during growth more rapidly when they are presented as such than when it is necessary for the organisms to synthesize them from nicotinic acid. To investigate this, it was attempted to apply the observation of Mann and Quastel (76) that nicotinamide inhibited the breakdown of coenzymes in animal tissues. No synergism in growth effects between cozymase and nicotinamide was observed, when each was used at levels of about  $10^{-8}$  *M* (82). This experiment is however inconclusive, as the concentrations of nicotinamide found effective by Mann and Quastel were over  $10^{-3}$  *M* and the reaction in animal tissues may be of a different type from that in *P. vulgaris*. The possibility that differential activity in growth is conditioned by different rates of inactivation therefore remains open.

Whatever the explanation of the lesser activities of the coenzymes, the observation that their lesser activity in growth parallels that found in their metabolic stimulation is of value in emphasizing that the two processes are closely interdependent. A further correlation of this type is afforded by the observation (47) that nicotinic acid is unable to replace nicotinamide, both in growth and in hydrogen transport (from glucose to methylene blue), by *Pasteurella suisseptica*. The acid can replace the amide in both activities in related organisms (102).

The concentrations of nicotinic acid derivatives required for optimal growth response have been found to be rather lower than, but of the same order of magnitude as, those required for maximal stimulation of respiration. Dysentery bacilli gave optimal growth in one day with about  $10^{-7}$  *M* nicotinamide (15,16,48) while this gave less than one-fifth the maximum rate of oxygen uptake with glucose as substrate (14,102). Presumably the growth was being limited by factors other than nicotinamide. It was in a

"synthetic" medium, but the lag or rate of growth, which might indicate the adequacy of the medium, was not recorded. Growth with the minimum quantity of nicotinamide optimal for growth yielded cells whose respiration could be stimulated by added nicotinamide.

**Actions of Some Inhibitors.** Pyridine-3-sulfonic acid inhibits the growth of strains of *P. vulgaris* in a manner suggesting that its action is related to the organisms' response to nicotinic acid (62, see also 19,20,80, 82). One strain required  $1$  to  $2 \times 10^{-6}$  *M* nicotinic acid or amide for its maximum growth in a medium of ammonium lactate and inorganic salts (28), and about  $1.6 \times 10^{-8}$  *M* for just visible growth. No considerable difference was shown in its response to the two compounds under these circumstances, but growth in the same media after addition of pyridine-3-sulfonic acid showed a marked difference in the organisms' relationship to nicotinic acid and amide (62). When growth was attempted with concentrations of nicotinic acid up to  $10^{-5}$  *M* in the presence of  $10^{-2}$  *M* pyridine-3-sulfonic acid, visible growth did not appear until the third day after inoculation; the excess represented by  $10^{-5}$  *M* nicotinic acid did not hasten growth in the presence of the sulfonic acid, but slightly reduced its amount. The effect of the sulfonic acid could, however, be overcome by substitution of nicotinamide for nicotinic acid; growth of the organism in the presence of  $10^{-5}$  or  $10^{-6}$  *M* nicotinamide and  $10^{-2}$  *M* pyridine-3-sulfonic acid was equal to or greater than its growth with nicotinamide alone.

It thus appeared that the action of pyridine-3-sulfonic acid was localized at a reaction converting nicotinic acid to its amide, or at a similar stage of amide formation in a higher derivative. From this point of view, the action of cozymase as an antagonist to pyridine-3-sulfonic acid was unexpected, for the compound was less effective as antagonist than either the acid or amide; but analogous phenomena have been discussed on page 417.

Following their finding that nicotinamide-promoted respiration of dysentery bacilli was inhibited by sulfapyridine or sulfathiazole, Dorfman and Koser (13) examined the possibility that inhibition of growth, also, by these compounds might be related to nicotinic acid derivatives. No antagonism by nicotinamide or cozymase was observed; systems involving *p*-aminobenzoic acid are presumably more critical in growth. Some antagonism by coenzyme preparations in staphylococci has been reported (121).

#### **Rate of Inactivation of Nicotinamide Derivatives and Rate of Growth.**

Inactivation in the presence of glucose was at the rate of 15% per hour of the nicotinamide of the cell (page 419). This may be "wearing out"

incidental to functioning (similar to that discussed on pages 412 and 419) but, in attempting to assess its relationship to the economy of the organisms as a whole, the rate of growth of the cultures concerned (81) was calculated and found to be such that the population doubled every three hours. This is a slow rate of growth for coliform organisms; but the present growth was at 30°C. in very simple media. It will thus be observed that the culture if growing could double in size during the time in which it inactivates about half the nicotinamide associated with it. It would appear not impossible that this quantity of nicotinamide may be diverted during growth to other uses of importance to the bacteria in connection with the synthesis of new cell materials. Such considerations emphasize the necessity of investigating the fate of the inactivated nicotinamide.

### C. NICOTINIC ACID DERIVATIVES AND VARIOUS MICROORGANISMS (ESPECIALLY YEASTS)

#### 1. *Stimulation of Metabolism by Coenzymes*

The role of coenzymes I and II in many processes of yeasts has been studied sufficiently for quantitative details of interactions of the pure compounds with crystalline or highly purified enzymes to be available in books of reference and reviews (34,91,103,104). No account of such reactions is attempted here. The synthesis and degradation of the coenzymes in yeast is, on the other hand, much less clearly defined and forms the main subject of the present account.

#### 2. *Metabolism of Nicotinic Acid*

Steps in the synthesis of the nicotinic acid nucleus in microorganisms have not yet been specified. The only related compounds not containing the pyridine nucleus, which have been recorded as replacing nicotinamide in microorganisms, are the tetrahydro (guvacin) and hexahydro derivatives, which support growth of *Proteus vulgaris* (25). Knight (46) observed that all bacterial cultures grown without nicotinic acid derivatives, which have been examined for the presence of such compounds, have been found to contain them. Study of the quantities of nicotinic acid and its derivatives (determined after hydrolysis as nicotinic acid) synthesized by bacteria has been confined to observations in growing cultures (10,114), and shows that bacterial species differ markedly in the quantities of nicotinic acid derivatives which they form. *Bacillus vulgatus* yielded the equivalent of a solution  $10^{-5}$  M with respect to nicotinic acid while the corresponding value with *Escherichia coli* was  $2 \times 10^{-7}$  M. Nevertheless, the content in nico-

tinic acid of the harvested cells could be remarkably constant. The six species examined by Thompson (114) contained between 1.8 and 2.05  $\mu\text{M}$  per mg. dry weight although the concentrations in their culture fluids varied over a thirtyfold range.

Values recorded for *Aerobacter aerogenes* (114) suggest it inactivated added nicotinic acid during growth, at a rate comparable to that at which the compound was synthesized if not added. This may be comparable to the breakdown and synthesis of coenzymes found in yeasts (see below), to which its rate appears to approximate. An order of magnitude may be given to the rate, as follows: the changes are about 4  $\mu\text{M}$  of nicotinic acid derivatives by cultures yielding about 3 g. of moist cells after 24 hours at 33°C.; if this implied 600 mg. dry weight of cells acting for 10 hours, the rate would be  $\pm 0.8 \mu\text{M}$  per mg. dry weight per hour.

A reaction with nicotinic acid, very different in rate, is its conversion to substances no longer containing the pyridine nucleus, by "bacillus N.C.," a Gram-positive pleomorphic organism isolated from soil (2). Here nicotinic acid could serve as main source of carbon and nitrogen to the organism (though nicotinamide was unattacked). From the partial data given, the rate of decomposition appears to be about 0.3  $\mu\text{M}$  per mg. dry weight per hour in resting suspension (assuming 1 ml. of culture to yield 1 mg. dry weight of cells).

### 3. Metabolism of Coenzymes I and II by Yeasts

The interconversion, breakdown, and synthesis of the coenzymes have received much more study in yeasts than have other reactions of nicotinic acid derivatives. In distinction to the studies of these compounds in other organisms, which have been recounted above, the quantities of substances reacting in yeasts have in many cases been estimated in defined enzyme systems after separation from reaction mixtures (58,104). The methods are sensitive to the quantities of coenzymes associated with a few milligrams of microorganisms.

Some details may be given of the apozymase system, as it is one not only used for estimating cozymase but also one in which its metabolism has been extensively studied. Cozymase becomes a limiting factor in alcoholic fermentation by yeast when this is dried and repeatedly washed with water, leaving "apozymase." Apozymase, with phosphate, hexose diphosphate, glucose, magnesium, and manganous salts, produces carbon dioxide at a rate proportional to added cozymase when the quantity of that is below about 0.15  $\mu\text{M}$  per mg. of apozymase. Such systems have only a limited ability to transform coenzyme II to cozymase (see below; and 3,56,85). Cozymase has been determined also by its effect in the dismutation of hexose diphosphate to 3-phospho-

glyceric acid and glycerophosphoric acid, brought about by a muscle preparation in the presence of arsenite, the additional acidic group formed being determined manometrically by evolution of carbon dioxide from bicarbonate (44). Coenzyme II has been estimated by its effect on oxidation of hexose monophosphate by an enzyme from red blood corpuscles or yeast (1,119). The oxidation has been followed by determining the rate of reduction of methylene blue using the Thunberg technic, or spectroscopically by determining the extinction coefficient of the reaction mixture at 334 m $\mu$ , the wave length of maximum absorption of the characteristic band of dihydrocoenzyme II. In addition, added coenzymes can be separated from reaction mixtures in which some 40 mg. of the substances has been added to purified enzymes, by precipitating associated materials with lead and the coenzymes with silver salts (77).

#### 4. *Conversion of Coenzyme II to I*

Considering first the breakdown of coenzyme II, it was observed (22,23) that the coenzyme specificity of the yeast apozymase system was not entirely confined to cozymase, but that coenzyme II could function after an induction period. This period represented the time required for production of an active agent, presumably cozymase; for, if the reaction solution was separated during the period of rapid reaction and added to a fresh apozymase system, this became active immediately without any lag. The cozymase was characterized also by its action in the lactic dehydrogenase system of muscle, but was not isolated. The yield, judged by activity in the apozymase system, was 12–47% of the added coenzyme II; it is not reported whether the remainder of this substance was left unchanged at the end of the reaction, but observations reported below suggest an equilibrium between the two coenzymes to be established during fermentation.

The rate of conversion of coenzyme II to I to some extent followed the degree of fermentation in the apozymase system; both processes required hexose diphosphate and were less rapid aerobically than anaerobically. Muscle adenylic acid retarded the conversion. Its rate in the presence of glucose, hexose diphosphate, and phosphate at pH 6.4 (30°C.) calculated from the data of von Euler and Adler (22) is approximately 0.4 m $\mu$ M per mg. apozymase per hour.

#### 5. *Conversion of Coenzyme I to II*

This conversion has been observed in the apozymase system under conditions similar to those under which the reverse change occurs. It takes place also in an aqueous extract ("maceration juice") from dried ground bottom yeast, while this is catalyzing the oxidation of hexose diphosphate (1,24). Not any oxidation in which cozymase is involved is adequate for conversion of cozymase to coenzyme II; the preparation oxidized alcohol

without such conversion. Nor did the progress of the conversion follow the progress of oxygen uptake in the oxidation of hexose diphosphate.

The following estimate of the rate of the reaction may be made. 1 ml. of maceration juice prepared by extracting dried yeast with three volumes of water (equivalent therefore to 330 mg. yeast, ignoring volume changes during a subsequent dialysis) produced about  $0.3 \mu\text{M}$  of coenzyme II in two hours at  $30^\circ\text{C}$ . (1). This approximates a change of  $0.5 \text{ m}\mu\text{M}$  per mg. dry weight per hour. During a similar experiment oxygen uptake proceeded at about 100 times this rate.

These observations gave the impression that conversion of the coenzymes was connected with phosphorylated intermediates in hexose diphosphate breakdown rather than with dehydrogenation, and this was supported by the action of inhibitors on the conversion in the maceration juice. Iodoacetate inhibited the reaction completely. The stage in fermentation most sensitive to this compound is that of triose phosphate dehydrogenation, which in yeast concerns 3-phosphoglyceraldehyde, coenzyme I, and phosphate, yielding 1,3-diphosphoglyceric acid and the reduced coenzyme (118). The 1,3-diphosphoglyceric acid is then capable of phosphorylating adenosine diphosphate to the triphosphate. Its ability to interconvert the coenzymes does not appear to have been examined.

Fluorides and arsenites each inhibited the coenzyme conversion only partially, but together could inhibit it completely (1); this suggested two independent mechanisms for the conversion. Fluoride did not affect triose phosphate dehydrogenation but inhibited the conversion of phosphoglyceric acid to phosphopyruvate. To elucidate the fluoride-sensitive system converting coenzyme I to II, 3-phosphoglyceric acid was itself used as substrate with yeast maceration juice. The reaction, however, proceeded beyond the formation of phosphopyruvic acid, and was accompanied by carbon dioxide formation. The fermentation of phosphoglyceric acid was not as rapid as that of hexose diphosphate by the same maceration juice, but was accelerated by both added phosphate and cozymase. During the reaction a progressive conversion of cozymase to coenzyme II occurred, at an initial rate which (making the assumptions noted above in calculating the corresponding value with hexose diphosphate as substrate) corresponded to  $1.8 \text{ m}\mu\text{M}$  per mg. yeast per hour. Fluoride completely inhibited this reaction. During the first hour the course of formation of coenzyme II roughly paralleled the course of carbon dioxide evolution, but the quantities of substances involved were very different; about 100 moles carbon dioxide were evolved per mole of coenzyme formed. The quantities became more divergent during the later part of the experiment and the

yield of coenzyme II was about 10% of the cozymase added. As the reaction proceeded beyond the stage of pyruvate, opportunity was given for a further transfer of energy-rich phosphate from phosphoenolpyruvate, which is capable of phosphorylating adenosine diphosphate to the triphosphate.

Further results showed a certain, but again limited, parallelism between the rate of carbon dioxide evolution from phosphoglyceric acid and the rate of production of coenzyme II (1). Carbon dioxide evolution was increased by magnesium and manganese salts, as also was the quantity of coenzyme II produced. Arsenite strongly inhibited the coenzyme formation in this system also, either with or without the manganous and magnesium ions, but in the former case carbon dioxide evolution was accelerated, and in the latter case it was inhibited.

Many reactions in yeast preparations can thus lead to conversion of coenzyme I to II. A single reaction which may be common to all the above systems and be the immediate source of coenzyme II has not yet been specified. Von Euler and Bauer (24; see also 26,27,115) found adenosine triphosphate necessary for the conversion in a preparation from "maceration juice"; but in this system also a major reaction was proceeding. The preparation consisted of material precipitated by carbon dioxide from the yeast extract and dialyzed (117); the systems discussed above have not required added adenosine triphosphate and have presumably contained such adenosine derivatives.

### *6. Inactivation of Coenzymes*

The following paragraphs show that the progress of metabolic reactions of micromolar order can condition not only the balance between coenzymes I and II but also the maintenance of the total coenzyme content of yeasts.

**Inactivation of Cozymase in the "Pyocyanin System."** The usual apozymase system (page 424) will ferment glucose with production of carbon dioxide for some hours. In the presence of fluorides, phosphorylation of glucose still occurred, and this and oxygen uptake were considerably accelerated by pyocyanin ( $3 \times 10^{-4} M$ ); but after these additions (constituting the "pyocyanin system" of Lennerstrand; 56,58) both processes soon slowed and gradually ceased. This was found to be due to an inactivation of the cozymase, for on its addition the oxidation and phosphorylation rapidly recommenced; other components of the reaction mixture, and also adenosine triphosphate or adenylic acid, did not have such effect.



When cozymase was added to the system without pyocyanin, oxygen uptake was much smaller, but could be restored to its usual value if pyocyanin was added fairly promptly. If cozymase was present but addition of pyocyanin was delayed, its effect became less but could be increased to its previous magnitude by cozymase. Thus cozymase inactivation was occurring independently of the presence of pyocyanin. The addition of fluoride, therefore, had so modified glucose breakdown that some reaction protecting or restoring cozymase was affected. The quantity of added hexose diphosphate also conditioned the duration of oxygen uptake and phosphorylation, and a large quantity of it in the pyocyanin system to some extent protected cozymase; but addition of hexose diphosphate after inactivation of cozymase did not restore its activity.

The initial rate of oxygen uptake by the pyocyanin system containing limited quantities of cozymase (0.15 to 1.5  $m\mu M$  per mg. apozymase) was proportional to the quantity of added cozymase, being about 14  $\mu M$  per hour with 0.3  $\mu M$  of cozymase, equivalent to about 100 moles hydrogen per mole cozymase per hour. Although this is low in comparison with the response of the apozymase system to cozymase, Lennerstrand (58) has made considerable use of the pyocyanin system in estimating cozymase. When the above quantity of cozymase was used with 0.2 g. apozymase, the  $Q_0$  of the apozymase was about 0.1  $\mu M$  per mg. per hour, but its maximum value was about three times that value (57).

The later course of oxygen uptake in the pyocyanin system fell exponentially with time, as did also the rate of cozymase inactivation. This is a reflection of the dependence of the inactivation upon the progress of reactions in which cozymase itself plays a necessary part, and whose rate is proportional to the quantity of cozymase available. It can be deduced mathematically and was found experimentally that the total oxygen uptake of such a system is proportional to the quantity of added cozymase. Some 30 moles of oxygen were evolved for each mole of added cozymase. The initial rate of inactivation of cozymase in the reaction mixture of the preceding paragraph was about 3  $m\mu M$  per mg. apozymase per hour—which is much more rapid than that of its conversion to coenzyme II (see page 426).

**Inactivation by Apozymase Alone.** Inactivation of cozymase was first studied in some detail in the pyocyanin system because it was made evident by the transitory effect of cozymase on oxygen uptake by the system; but a modified experimental arrangement showed that the inactivation was brought about by apozymase alone, though with character-

istic differences. In this case it was necessary for experiments to consist of two parts: (1) incubation of apozymase with cozymase and (2) either the completion of the pyocyanin system, or else heating the reaction mixture which had contained cozymase to inactivate and precipitate its apozymase, followed by assay of the product in a separate reaction mixture. When such experiments were arranged so that the kinetics of the inactivation could be followed, inactivation was found to commence after a brief lag and to continue at a steady rate until almost all added cozymase had reacted. The type of inactivation was thus different from the exponential course found in the pyocyanin system, but its rate was comparable to the initial rate found in that system, data given by Lennerstrand (58) indicating a rate of about 3  $\mu$ M per mg. apozymase per hour.

Identification of the products from cozymase has not been reported, but certain possible ones have been investigated. Use of the hexose monophosphate system showed no coenzyme II to be formed; hexose diphosphate has been shown to be necessary for this conversion. Evidence regarding the production of ammonia during inactivation was sought but the findings were inconclusive (58).

More extensive studies were made of the influence of added substances on the course of cozymase inactivation. This was normally performed at pH 6.2 but was unaffected by variation in pH between 5.6 and 6.8. Nicotinamide, which protects cozymase in other systems (76), did not protect it in apozymase, but the concentration used ( $5.5 \times 10^{-3}$  M) was relatively low. Increase in phosphate concentration from  $M/30$  to  $M/6$  afforded partial protection, as also did adenosine triphosphate and muscle adenylic acid; yeast adenylic acid, adenosine, and adenine were without effect. Hexose diphosphate protected cozymase, and fluoride antagonized the protection; as fluoride had no effect on the stability of cozymase in the presence of apozymase alone its effect on cozymase was presumably secondary to the effect on hexose diphosphate breakdown. Of breakdown products from hexose diphosphate, 3-phosphoglyceric acid and 2,3-diphosphoglyceric acid did not protect cozymase; glucose not only did not protect, but also prevented hexose diphosphate from protecting cozymase.

Cozymase stability thus appeared to be associated with phosphate transference. Oxalate, which inhibits breakdown of phosphopyruvic acid and of monophosphoglyceric acid, inhibited cozymase inactivation. Experiments aimed at directly observing changes in phosphate associated with cozymase inactivation were inconclusive because other changes in phosphate were occurring: the apozymase used liberated inorganic phosphate

on exposure in water at 25°C., and cozymase during its inactivation decreased the liberation (58).

### *7. Reactivation of Cozymase*

The stability of cozymase during ordinary fermentation in yeast is thus clearly associated with the progress of the fermentation, but whether it is dependent on inhibition of systems normally inactivating cozymase, or upon resynthesis of cozymase, has not been determined by the preceding experiments. Its resynthesis is made probable by the following findings which thus take the study of cozymase in yeast to a further stage than it has been taken in other microorganisms.

When cozymase was treated with apozymase until the pyocyanin system indicated a large part of its activity to have been lost, very little carbon dioxide was evolved within an hour or two of completing the apozymase system by adding glucose, hexose diphosphate, and phosphate. After this period a gradually accelerating evolution of carbon dioxide was observed (58). The speed of reactivation of the apozymase system was accelerated by muscle adenylic acid and was slowest when the initial cozymase inactivation was most complete. Material from the reactivated system behaved as cozymase in the more specific pyocyanin system. It was not chemically characterized as cozymase; such characterization is desirable as only about 15% of the cozymase activity which had been lost was restored in this way.

Under different conditions, restoration of cozymase activity was much more complete. Fermentation by apozymase with glucose, hexose diphosphate, muscle adenylic acid, and cozymase, but without inorganic phosphates, soon ceased and this was shown to be due to inactivation of the cozymase. Addition of inorganic phosphates when carbon dioxide evolution by the mixture had become negligible, led to a gradual recovery of fermentation to about 60-70% of its initial rate. This took about three hours. Analysis of the course of reactivation showed it to be mainly exponential, indicating the resynthesis of cozymase to depend upon the occurrence of the fermentation which it was itself catalyzing. Reactivation was less complete the longer the system was left without phosphate, suggesting occurrence of secondary changes in the primary products from cozymase. The speed of reactivation was 0.6  $\mu\text{M}$  per mg. apozymase per hour at a time when carbon dioxide evolution was proceeding at 75  $\mu\text{M}$  per mg. per hour, which was about one-third to one-fourth its maximum

value in the system concerned; during its exponential course, the activity of the cells was doubling each 80 minutes.

It has further been shown that the processes of inactivation and reactivation occur not only in the relatively artificial systems prepared from apozymase, but also in the cozymase which is naturally associated with fresh and viable yeasts.

Bakers' yeast shaken in aqueous suspension usually increased in cozymase content during 24 hours. The cozymase was estimated in the pyocyanin system after extraction from the yeast by heating at 80–85°C. If, however, exposure of the yeast was in fluoride-containing solutions, fall in cozymase content was soon evident and the loss reached 78–92% after 23 hours;  $M/60$  fluoride produced evident inactivation and  $M/6$  gave a maximum effect. The approximate rate can be calculated from data of Lennerstrand (58). Supposing the quantity of yeast quoted to refer to moist weight of material with 80% water, and the method of extraction to yield all the cozymase (Cheldelin *et al.*, 11, found nicotinic acid derivatives easily extracted from yeasts), then two sets of data indicate losses of 0.3 and of 0.65  $m\mu M$  cozymase per mg. dry weight per hour. The latter value represents inactivation of about one-fifteenth of the yeast's cozymase per hour.

The products of inactivation remained associated with the cells. If the yeast whose coenzyme had been mostly inactivated was separated by centrifuging, washed free from fluoride, and incubated in aqueous suspension, its cozymase activity increased, a large part being recovered after 2 hours and the majority after 23 hours (58). Thus the irreversible inactivation occurring in apozymase did not take place in fresh yeast; or, this was capable of recovery processes of which the apozymase was not. After fluoride treatment and washing the yeast remained viable. Examination of the course of its growth has not been reported but would appear to afford a valuable opportunity of studying the effect of a specific reaction on the character of growth.

Though in this system also the products of inactivation of cozymase have not been determined chemically, the ability of components of the cozymase molecule to serve as precursors of the substance has been demonstrated. Cozymase production by yeast in aqueous suspension at 25°C. for some 22 hours was markedly increased by added adenine, adenosine, or nicotinic acid, and still further by mixtures of these or by glucose. Kinetic experiments comparing the production of cozymase from such substances with production from the unknown products of inactivation were not reported, results quoted (58) referring to cozymase extracted from the cells at one time only; but the rates of production are unlikely to differ greatly. The spontaneous reactivation at 25° was at a rate of about 2.5  $m\mu M$  per

mg. dry weight per hour during its initial phases, when about 20% of its cozymase content was reappearing per hour. This rate is greater than that of the inactivation induced by fluoride.

TABLE I  
RATES OF MICROBIAL REACTION WITH NICOTINIC ACID AND ITS DERIVATIVES

Organism or preparation (reference)	Reaction mixture	Change in nicotinic acid or derivative*	
		Reaction	Rate, $\mu\text{M}/\text{mg.}$ dry wt. of organism/hr.
"Bacillus N.C.," non-proliferating suspensions (2)	Phosphates; pH 7.6; 37.5°	Decomposition of nicotinic acid	-300
<i>A. aerogenes</i> , growing in simple media (114)	Glucose, phosphates, and inorganic salts; 33°	Inactivation and synthesis of nicotinic acid	$\pm 0.5$
<i>P. vulgaris</i> , in culture during and after growth (81,82)	Glucose, phosphates, and inorganic salts; pH 7.6; 37°	Inactivation of nicotinamide	-0.3
<i>H. parainfluenzae</i> , non-proliferating suspensions (61)	Glucose and phosphates; pH 7.5-6.8; 38°	Inactivation of cozymase	-0.6
Yeast (bakers', fresh), nonproliferating suspension (58)	Aqueous suspension with fluoride; 25°	Inactivation of cozymase	-0.3 to -0.65
	Aqueous suspension, after incubation with and removal of fluoride; 25°	Reactivation of cozymase	+2.5
Yeast (apozymase) (58)	Glucose, phosphate, hexose diphosphate, fluoride, and pyocyanin; 25°	Inactivation of cozymase	-3
	Yeast after inactivation of cozymase, with glucose, phosphate, hexose diphosphate, and muscle adenylic acid; 25°	Reactivation of cozymase	+0.6
Yeast (maceration juice) (1)	Phosphoglyceric acid and phosphate; pH 7.15; 30°	Conversion of cozymase to coenzyme II	+1.8
	Hexose diphosphate and phosphate; pH 7.15; 30°	Conversion of cozymase to coenzyme II	+0.5
Yeast (apozymase) (22)	Glucose, hexose, diphosphate, and phosphate; pH 6.4; 30°	Conversion of coenzyme II to cozymase	+0.4

\* Many of these values are based on estimates, made by the author and given in detail in the text, of the dry weights of organisms or preparations which were employed by the various investigators.

## D. SURVEY OF REACTIONS IN NICOTINIC ACID AND ITS DERIVATIVES

Nicotinic acid derivatives thus occur in all microorganisms examined, in similar quantities; and in the organisms studied they participate in similar processes of hydrogen transport. Equally widespread are reactions of the millimicromolar order which result in their formation, interconversion, or inactivation (Table I). The much more rapid reaction by "bacillus N.C.," which derives its main material and energy from nicotinic acid, emphasizes the similarity between the rates of the other reactions.

The dependence of such reactions on processes of the micromolar order is of varying degrees. In assessing such dependence, reactions of viable yeast must at present be excluded in view of the uncertain part played by stored metabolites; the clearest evidence of connection between reactions of the micromolar and millimicromolar orders appears to be that given by their exponential course as the minor constituent is activated or exhausted. Processes of inactivation showing this feature include those concerning nicotinic acid and cozymase in *Proteus vulgaris* and *Hemophilis parainfluenzae*, and those in yeast, as apozymase, in the presence of fluoride, pyocyanin, and hexose diphosphate. Whereas a type of inactivation unconnected with reactions of micromolar order has not been observed in the first two organisms, it is well defined in apozymase alone. The additions to apozymase, which constitute the pyocyanin system, have thus imposed upon or revealed in the yeast a type of linkage which exists naturally in the other two organisms. These two differ from yeast in their need for added nicotinic acid or its derivatives in their growth. Schemes embodying this data can be devised, but must remain largely hypothetical until the nature and further properties of the products of inactivation are known.

Reactivation of cozymase by apozymase is not spontaneous; it and the reciprocal conversion of coenzymes I and II require concomitant reactions (Table II) of which, in common with reactions associated with cozymase inactivation, the main feature appears to be phosphate transfer. This does not necessarily support suggestions that the product of inactivation of cozymase may be a dephosphorylated derivative; conversion of glutamine to glutamic acid and  $\text{NH}_3$  by streptococci is similarly conditioned (69,71).

Another concept of the inactivation of nicotinamide derivatives which requires cautious application is the suggestion of Lwoff and Lwoff (61) and Morel (81,82) that inactivation is a process of attrition, necessarily associated with the functioning of the substances as coenzymes, and that it is a faulty type of reaction taking place as an accident on a few out of the many

TABLE II

COMPARISON BETWEEN RATES OF REACTIONS OF NICOTINIC ACID DERIVATIVES  
AND THOSE OF ASSOCIATED METABOLIC PROCESSES\*

Organism or preparation (reference)	(a) Reaction with nicotinic acid derivative		(b) Associated reaction		Ratio of rates (b/a)
	Process†	Rate, $\mu\text{M}/\text{mg. dry wt./hr.}$	Process	Rate, $\mu\text{M}/\text{mg. dry wt./hr.}$	
<i>P. vulgaris</i> (81, 82)	Inactivation of nicotinamide	-0.3	Uptake of oxygen or reduction of methylene blue in presence of glucose (expressed as moles of hydrogen)	36	120,000
<i>H. parainfluenzae</i> (61)	Inactivation of cozymase <sup>a</sup>	-0.6	Reduction of methylene blue in presence of glucose	7.2	12,000
Yeast (apozymase) (58)	Inactivation of cozymase	-3	Uptake of oxygen in "pyocyanin system"	0.1	33
	Reactivation of cozymase	+0.6	Carbon dioxide evolution in apozymase system	0.075	125
Yeast (maceration juice) (1)	Conversion of cozymase to coenzyme II	0.5	Uptake of oxygen with hexose diphosphate	0.05	100
	Conversion of cozymase to coenzyme II	1.8	Carbon dioxide evolution with phosphoglyceric acid	0.18	100
Yeast (apozymase) (22)	Conversion of coenzyme II to cozymase	0.4	Carbon dioxide evolution with glucose and hexose diphosphate	0.38	1000

\* Values have been computed by methods given in the text.

† Further details in Table I.

occasions (1 in  $10^4$  or  $10^5$ ) during which the substances undergo their cyclic hydrogenation and dehydrogenation. Certainly, any but a perfect catalyst must suffer attrition, and in autotrophic organisms processes of resynthesis with velocities comparable to those of the degradations are to be expected. Consideration of coenzyme inactivation in the wider range of systems of the present review shows however that its rate, in  $\mu\text{M}$  per mg. dry weight per hour, is a much more constant feature than is its association with a micromolar process. The rates of Table I show very marked similarity; the ratios of Table II, a wide variation. Moreover, the inactivation

is one of a number of processes of similar speed which involve the co-enzymes, and the micromolar processes with which these are associated again vary considerably in speed (Table II). These observations limit rather than discredit the Lwoffs' hypothesis and suggest the uniformity in character of reactions of Table I requires investigation. Many assumptions have been made in the calculations, and it is impossible to say whether the exchange of  $3 \text{ m}\mu\text{M}$  per mg. dry weight per hour in yeast preparations includes perhaps  $0.5 \text{ m}\mu\text{M}$  per mg. dry weight per hour due to "attrition."

A more definite and important outcome of the computations concerns the physiological significance of the rates. Taking  $2 \text{ m}\mu\text{M}$  per mg. dry weight as a typical microbial content of nicotinic acid derivatives, reactions with velocities of  $0.5$  to  $3 \text{ m}\mu\text{M}$  per mg. dry weight per hour result in the turnover of quantities of the substances comparable to those of the cells, in times approximating those required for the production of such cells. This may be further connected with the aspect emphasized by Lwoff and Morel. More accurate comparison of the rates of metabolism and growth in specific organisms are only occasionally available (see page 422) but suggest investigation of the subject to be practicable and of value. That this is a relatively novel outlook is emphasized by the manner in which the metabolic quotients of Tables I and II have been based necessarily on estimates, in the absence of adequate recorded data.

### III. Pantothenic Acid

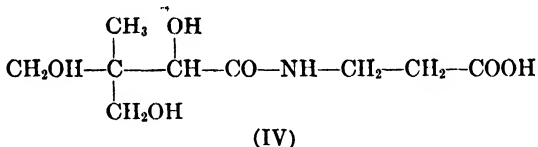
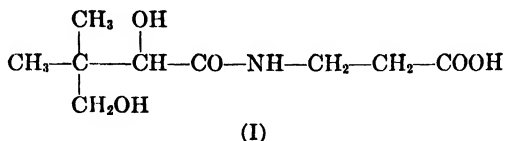
Knowledge of the existence and nature of pantothenic acid mainly developed in connection with microorganisms, and is more recent than that of the biological properties of nicotinamide (125). In contrast to the account given of nicotinamide derivatives, more is known in the case of pantothenate of its metabolism in bacteria, but much less of the manner of its functioning.

#### A. MICROBIAL SYNTHESIS OF PANTOTHENIC ACID

As is the case with other growth essentials, much indirect evidence concerning the metabolism of pantothenate is available from experiments studying the nutrient needs of microorganisms. These show, for example, that some organisms need pantothenic acid (I) as such for their growth while others can grow when given parts of the molecule, or when given only unrelated nutrients. All the organisms examined have been found to contain pantothenate. They have therefore synthesized it, in recorded instances either from pantoic acid and  $\beta$ -alanine, or glucose and amino acids,



or ammonium lactate (46,93). The chemical nature of pantothenic acid (I) suggests  $\beta$ -alanine (III) and pantoic acid (II) as intermediates in its production from other sources, as also do biological properties of those two compounds.



Promotion of growth by a substance differing little from the intact pantothenate molecule is susceptible of two alternative explanations: that it may act as such, or after conversion to pantothenate. The question of this type raised by the activity of *N*-( $\alpha$ -hydroxy- $\beta,\beta$ -dimethylolbutyryl)- $\beta$ -alanine (IV) (hydroxypantothenic acid, 79) has not been answered. The alternatives are particularly interesting as in different organisms the ratios between the activities of pantothenic acid and hydroxypantothenic acid vary considerably, representing either different powers of the organisms in converting the substance to pantothenate, or different abilities to use it as such in place of pantothenate. Inactive substances may immediately be concluded to be neither capable of reacting adequately with systems requiring pantothenate, nor capable of conversion to pantothenate.

**Origin of  $\beta$ -Alanine.** Virtanen and Laine (116) isolated  $\beta$ -alanine as the product of decarboxylation of aspartic acid by nonproliferating suspensions of *Rhizobium leguminosarum*. The process was a slow one but an approximate calculation of its rate shows this to be comparable with the rate of microbial reactions involving pantothenate (see page 439). An approximately linear evolution of carbon dioxide was observed during 40 days at about 20°C. The total carbon dioxide evolved approximated the theoretical value and the rate of its evolution was about 0.14 mM per day by organisms from five Roux bottles; supposing this to yield 0.2 g. dry

weight the rate of the reaction would be 30  $\mu\text{M}$  per mg. dry weight per hour. Other bacterial amino acid decarboxylations can reach velocities of 12,000  $\mu\text{M}$  per mg. dry weight per hour (30).

Decarboxylation of aspartic acid presumably does not occur in certain yeasts which require  $\beta$ -alanine for growth in media containing aspartic acid (101) nor in strains of *Corynebacterium diphtheriae* which require added  $\beta$ -alanine for growth in the presence of a casein hydrolyzate (84). Of the latter organisms, some were able to utilize *l*-carnosine (but not the *d*-isom-eride) in place of  $\beta$ -alanine, but this resulted in a delay in their growth which did not occur when  $\beta$ -alanine was used. The quantities required of  $\beta$ -alanine were small and its production from carnosine is also presumably of the order of some  $\mu\text{M}$  per mg. organisms per hour (83).

**Pantoic Acid.** A fermenting mixture of yeast, glucose, and phosphate has been found to produce the (—)-acid from  $\alpha$ -keto- $\beta,\beta$ -dimethyl- $\gamma$ -butyrolactone (51). Inhibition of growth of a number of bacteria by  $3 \times 10^{-4}$  to  $10^{-2}$  *M* salicylate was antagonized by pantothenate and considered to be due to interference with the synthesis of the hydroxy acid moiety (42). Higher concentrations of salicylate appeared to act by protein denaturation and were not antagonized by pantothenate. The organisms inhibited by the lower concentrations were those placed under circumstances requiring synthesis of pantoic acid, for example, *Escherichia coli* and *Proteus vulgaris* in simple media. These and other organisms growing from the preformed pantoic acid or pantothenate were not inhibited by the lower concentrations of salicylate.

**Synthesis of Pantothenate.** Inhibition of the growth of yeasts from  $\beta$ -alanine by aspartic acid (89,90) and by asparagine (101,120) has been ascribed to inhibition of the synthesis of pantothenic acid from  $\beta$ -alanine. In the latter instance inhibition with several strains could be prevented by higher concentrations of  $\beta$ -alanine or by replacing  $\beta$ -alanine by pantothenic acid (101). Inhibition of growth of yeasts from  $\beta$ -alanine was caused also by  $\beta$ -aminobutyric acid, and a similar explanation has been given (38,88). Systems concerned in the synthesis have been studied (124).

Observations on the production of pantothenate by bacteria have rarely given data adequate for calculation of production rate. The substance is frequently found after growth, both in the culture fluid as well as in the cells. In a comparative study of six organisms (114), the cells after growth contained 0.3 to 1.5  $\mu\text{M}$  per mg. dry weight, and the media contained from one-fourth to four times the quantities present in the cells. An order of magnitude may be given to the rate of synthesis by supposing

that the organisms (about 3 g. moist weight) were acting for 10 hours. The total pantothenate produced varied from 0.4  $\mu\text{M}$  per mg. (assumed) dry weight by *P. vulgaris* to 3  $\mu\text{M}$  by *Aerobacter aerogenes* giving rates of production of 0.04 to 0.3  $\mu\text{M}$  per mg. dry weight per hour.

In the case of two bacterial species, the rates of synthesis of pantothenate by growing and nongrowing organisms have been compared (70).

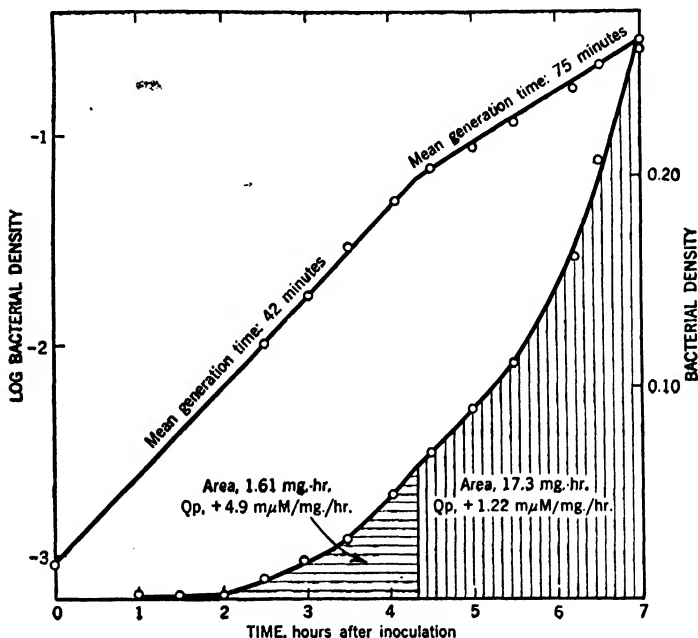


Fig. 1. Rate of pantothenate synthesis during growth of *E. coli* (69,71): lower curve, bacterial density; upper curve, log bacterial density.

The rate of synthesis in growing cultures was obtained by estimating pantothenate in portions of a culture at different times, and also measuring bacterial growth by optical density determinations. From the densities, a growth curve and logarithmic growth curve were constructed (Fig. 1). The logarithmic curve showed the phases through which growth was passing, while, from the areas (shaded) between portions of the growth curve and the time abscissa, the mg.-hr. of bacterial activity could be obtained. Synthesis of pantothenate ( $Q_p$ ) was then expressed in  $\mu\text{M}$  per mg. dry weight per hour during periods of growth of known characteristics. Mean generation time (MGT) is the time required for the culture to double in size.

During growth of the greater part of the organisms of cultures of *E. coli* and *Pseudomonas aeruginosa*, pantothenate synthesis occurred at rates between 0.7 and 1.6  $\mu\text{M}$  per mg. dry weight per hour. Synthesis occurred at this rate in nonproliferating suspensions of the organisms in similar media (Table III); when studied kinetically it was found to commence without lag and to proceed linearly for some 4 hours. In this it differed from the reaction during growth, for the latter rate was unusually high during the production of the first few  $\mu\text{M}$ . of pantothenate by the youngest organisms.

TABLE III  
RATES OF SYNTHESIS AND INACTIVATION OF PANTOTHENATE BY GROWING  
AND NONGROWING BACTERIA (70)

Organism	Nonproliferating suspension		During growth		
	Reaction mixture	Qp, $\mu\text{M}$ /mg. dry wt./hr.	Medium	Phases	Qp, $\mu\text{M}$ /mg. dry wt./hr.
<i>Escherichia coli</i>	Inorganic salts	+0.26	Inorganic salts with glucose	First 240 min. of logarithmic growth, MGT 61 min.	+7.0
	Inorganic salts with glucose	+2.8			
	Inorganic salts with glucose and $\beta$ -alanine	+30.3	Inorganic salts with glucose	Subsequent 80 min. of loga- rithmic growth, MGT 61 min.	+1.53
<i>Pseudomonas aeruginosa</i>	Inorganic salts	+0.04	Inorganic salts	Lag, 60 min.	+6.1
	Inorganic salts with lactate	+0.47	with lactate	First 180 min. of logarithmic growth, MGT 30 min.	
	Inorganic salts with lactate and $\beta$ -alanine	+0.57			
	Inorganic salts with lactate and pantoic acid	+1.67	Inorganic salts with lactate	Subsequent 60 min. of growth, MGT 30 to 108 min.	+0.73
<i>Proteus morganii</i>	Inorganic salts with glucose, casein hydroly- zate and a yeast prepara- tion	-1.41 to -6.9	As nonprolifer- ating suspen- sion	Logarithmic; MGT 26 to 42 min.	-3.8 to -6
$\beta$ -Hemolytic streptococci	Inorganic salts and glucose with or without ca- sein hydrolyzate and various growth essen- tials	-1.3 to -2.3	Inorganic salts with glucose, and casein hydrolyzate or broth	Lag, 90 min. Logarithmic growth, MGT 24 to 87 min.	-1.2 to -3.3

The rates in resting suspensions could, however, be increased by adding  $\beta$ -alanine or pantoic acid. Such precursors may have been present in the inocula; the higher production may also reflect necessary differences in the relation of bacteria in a very dilute suspension to their environment. The response of *E. coli* to  $\beta$ -alanine was especially large and the observations supported the general belief that  $\beta$ -alanine and pantoic acid are intermediates in the synthesis of pantothenate.

#### B. STATE AND QUANTITY OF PANTOTHENATE IN MICROORGANISMS

Some pantothenate may be loosely associated with bacteria after they have been grown in media containing a large excess of the substance, such pantothenate remaining associated with the organisms after washing at ordinary temperatures but capable of removal in salt mixtures at 37°C. (67). Organisms which have been grown with relatively little pantothenate, or which synthesize it and to which none has been added, contain less pantothenate but retain it more tenaciously. The residual pantothenate of pantothenate-rich organisms is similar in quantity and stability to that of the pantothenate-poor cells. In cell-free extracts from yeast, much pantothenate was found to remain attached to proteins in a non-dialyzable form (113). In this respect pantothenate of microorganisms behaves similarly to pantothenate from other sources; its initial isolation from animal tissues necessitated autolysis (127) and a protein derivative of pantothenate has been separated from liver (50).

As the liberation of pantothenate from organisms or tissues is a necessary preliminary to study of their content of the substance, the required conditions have been investigated in some detail with animal tissues and foodstuffs. The application of certain of these methods to microorganisms has been examined and found satisfactory (73,114; see also 43).

The concentration of pantothenate in bacterial cells varies considerably with different organisms, their growth media, and phases of their growth, and can be modified experimentally. The range of values obtained under normal conditions is illustrated in Table IV. When grown under uniform conditions, a variety of organisms yielded about 0.5  $\mu\text{M}$  per mg. dry weight but a given organism at different phases of growth could vary tenfold in its pantothenate content. The content did not markedly vary with the pantothenate of growth media except that in a given organism unusually high or low values were associated with excess or deficiency in media. This may be illustrated by a culture of *P. morganii* which at 5 hours contained 52  $\mu\text{M}$  pantothenate in 100 ml. fluid and 0.41  $\mu\text{M}$  in 4.2 mg. cells (0.1  $\mu\text{M}$  per mg. dry weight). At 7.5 hours, when growth and pantothenate inactivation (see page 444) had occurred, the

fluid contained 0.4  $\mu\text{M}$  and the 20 mg. of cells, 0.46  $\mu\text{M}$  (0.023  $\mu\text{M}$  per mg. dry weight). These cells were grown in media similar to that employed in producing cells of the same strain, which were shown by their metabolic behavior to be deficient in pantothenate. Computation of the quantity of pantothenate functioning in such cells has been made on page 443 on the basis of their metabolic response to pantothenate, with results which approximate those of direct determination.

TABLE IV  
PANTOTHENATE CONTENT OF BACTERIAL CELLS

Species	Conditions of growth	Pantothenate of culture fluid at time of harvesting, $\text{M} \times 10^{-6}$	Pantothenate of cells, $\mu\text{M}/\text{mg. dry wt.}$
<i>Aerobacter aerogenes</i>	24 hrs. at 33°C. in casein hydrolyzate with glucose, aerobic	ca. 1*	0.64
<i>Clostridium butylicum</i>	As above, anaerobic	0.6*	1.55
<i>Escherichia coli</i>	As above, anaerobic	0.3*	0.43
	Ammonium salts and lactate, 5.5 to 7 hrs., aerobic	0.26 to 0.90	0.05 to 0.66
<i>Proteus morganii</i> (laboratory strain)	Casein hydrolyzate with glucose and other additions, 37°C., aerobic, 3 to 5 hrs., pantothenate added	0.25 to 0.6	0.025 to 0.045
<i>Proteus morganii</i> (N.C.T.C. no. 2818)	As above, 5 to 7.5 hrs.	0.004 to 0.52	0.024 to 0.10
<i>Proteus vulgaris</i>	As <i>A. aerogenes</i> , aerobic	<0.05	0.46
<i>Pseudomonas aeruginosa</i>	As <i>E. coli</i> , 48 hrs.	0.17	0.07
<i>Pseudomonas aeruginosa</i>	Casein hydrolyzate with glucose at 37°C., 6 hrs., aerobic	0.32	0.095
<i>Pseudomonas fluorescens</i>	As <i>A. aerogenes</i> , aerobic	0.3*	0.42
<i>Serratia marcescens</i>	As <i>A. aerogenes</i> , aerobic	0.08*	0.55
$\beta$ -Hemolytic streptococci, groups A and G	Casein hydrolyzate with glucose and other additions, 37°C., aerobic, pantothenate added	<0.01	0.026 to 0.035
$\beta$ -Hemolytic streptococci, groups A and G	As above	ca. 1	0.17 to 0.72

\* These values are approximate only, being computed from data given (114) for the pantothenate of the culture fluids expressed as  $\gamma$  per g. dry weight of cells harvested, and the statement that wet yields of cells were about 3 g. per 2 liters of solution. Data for other organisms is from McIlwain (70,71).

#### C. BIOCHEMICAL ACTIVITIES OF PANTOTHENATE IN MICROORGANISMS

The metabolic role of pantothenate is not yet defined; but effects of deficiencies in pantothenate, on over-all processes of respiration and

fermentation in microorganisms, are relatively easily observed. Evidence pointing in this direction was obtained with yeasts, before pure pantothenate was available (97,126; see also 113). In more detailed studies of the metabolic effects of pantothenate *Proteus morganii* has been used, as its growth requirements are relatively simple but include pantothenate (92, 96). Cells grown with suboptimal concentrations of pantothenate (about  $10^{-8}$  M) were found less active in causing oxidation of several simple substrates than were cells grown with excess of the substance (e.g.,  $10^{-6}$  M) (12,39,40). Moreover the defective oxidation was made good by addition of pantothenate to washed nonproliferating suspensions of the cells, within a period of a few minutes. The mild hydrolysis which splits pantothenic acid to pantoic acid and  $\beta$ -alanine with loss of growth-promoting power, resulted in loss, also, of its metabolic action.

The effects of pantothenate on oxygen uptake (by *P. morganii*) in the presence of some twenty substrates have been examined (40). Of these, glucose, succinate, fumarate, pyruvate, lactate, and oxaloacetate caused most rapid oxygen uptake, and the oxidation of all except glucose was approximately trebled in rate by addition of excess pantothenate. This also had large effects (two- to three-fold increases) on oxygen uptake in the presence of glutamate,  $\alpha$ -ketoglutarate, aspartate, and alanine, and lesser effects in oxidation of malate, glycerol, galactose, acetoacetate, ethanol, and formate. Its action is thus not confined to a single substrate nor to a group of metabolically closely related substances.

In an attempt to limit reactions secondary to or associated with a pantothenate-sensitive system, the effects of pantothenate on anaerobic reactions were studied. When reactions were followed by evolution of gas from bicarbonate-containing solutions, only glucose, pyruvate, and oxaloacetate of those examined reacted appreciably. Here, as aerobically, the most rapid reaction of the organisms was with glucose but again pantothenate had little effect upon it. The action of pantothenate on the anaerobic production of carbon dioxide in the presence of pyruvate was outstanding, this reaction showing the largest stimulation (five- to seven-fold) of any examined aerobically or anaerobically. It was also taken much further toward completion.

The yield of carbon dioxide from the anaerobic reaction with pyruvate was about 0.75 mole per mole of pyruvate reacting, which was intermediate between those required for the two known bacterial reactions:  $2 \text{ pyruvate} \rightarrow \text{lactate} + \text{acetate} + \text{carbon dioxide}$  (49), and  $\text{pyruvate} \rightarrow \text{acetate}$  and formate (7,49). When fumarate was added, the yield became one

mole, suggesting the first reaction to be modified by fumarate acting as hydrogen acceptor in place of one mole of pyruvate. Oxaloacetate behaved similarly in this respect. The reaction was also increased in rate about threefold by fumarate, but the stimulation due to pantothenate was maintained. Addition of fumarate to other of the substrates studied anaerobically permitted reactions with  $\alpha$ -ketoglutarate, aspartate, oxaloacetate, and glycerol, all of which were accelerated by pantothenate but were much slower than the reaction with pyruvate.

Data given (40) for the effects of a graded series of pantothenate concentrations on reactions with pyruvate permit calculation of the turnover numbers of pantothenate in these systems. Though the systems are chemically not well defined, the numerical results are of value for comparison with other quantitative data concerning the metabolism of growth essentials. When the rates of the reactions are plotted against quantity of added pantothenate a well-marked initial period of linear response is shown in each case. From the slope of these lines the increase in rate of reaction, in moles per hour, brought about by one mole of pantothenate, can be calculated (Table V). The values are of the same order of magnitude as those for nicotinic acid derivatives in *P. vulgaris* and *H. parainfluenzae*.

The reactions of Table V in each case proceeded with definite velocity in the absence of added pantothenate. If it is supposed that these "blank"

TABLE V  
ESTIMATES OF TURNOVER NUMBERS OF PANTOTHENATE IN PYRUVATE METABOLISM,  
AND OF PANTOTHENATE CONTENT OF DEFICIENT CELLS\*

Substance measured	Change in substance (mole/hr.) catalyzed by 1 mole of pantothenate	Rate of change without added pantothenate, $\mu$ M/hr./mg. dry wt.	Pantothenate required to cause change, m $\mu$ M/mg. dry wt.
Carbon dioxide (including that liberated by acid from bicarbonate)	$+6.7 \times 10^4$	+0.76	0.011
Oxygen	$-2.3 \times 10^4$	-2.4	0.10

\* Data calculated from Hills (40).

values are due to the initial pantothenate of the cells, and that the turnover number of this is the same as that of pantothenate added later, the quantity of pantothenate associated with the cells can be calculated. The values yielded by estimation on the basis of the two reactions differ, being 0.1 and 0.01  $\mu$ M per g. dry weight of cells. Obvious explanations of this, which re-



main to be settled experimentally, are (a) that oxidation without added pantothenate is due to systems other than pantothenate-sensitive ones, and (b) that specific deficiencies in different systems requiring pantothenate are obtainable in a manner analogous to that in which *H. parainfluenzae* may become specifically deficient in coenzyme I or II. Values for bacterial pantothenate of 0.1 and 0.01  $\mu\text{M}$  per g. dry weight are in the same range as, or approximated by, the values directly determined (67,71) and quoted in Table IV; but in view of the manner of preparation of the deficient cells (40) the lower value appears the more probable. This favors suggestion (a) above.

It is evident that the metabolic potentialities of intact organisms make difficult the interpretation of the role of pantothenate and that examination of cell-free systems concerned with pyruvate from deficient organisms is necessary (40). Little use has been made of inhibitors, but dried *P. morganii* has been observed to have lost its ability to oxidize pyruvate. The ability to oxidize lactate remained, but whereas in intact cells it was stimulated some 80% by pantothenate, little or no stimulation was found in the dried preparation. The effect of pantothenate on lactate was presumably secondary to its effect on pyruvate (12).

#### D. BACTERIAL INACTIVATION OF PANTOTHENATE

Although bacteria requiring pantothenate usually reach optimal growth in media initially containing  $10^{-7}$  *M* or lower concentrations of the substance, the normal environments of parasitic or saprophytic organisms commonly contain higher concentrations of pantothenate. The behavior of microorganisms toward approximately  $10^{-6}$  or  $10^{-5}$  *M* pantothenate thus reflects more clearly their normal relationship to the substance than does their reaction to limiting quantities of it. This behavior has been found to be more active than would be suggested by the organisms' minimal requirements for pantothenate.

Pantothenate disappeared from solutions in which  $\beta$ -hemolytic streptococci, *P. morganii*, or *Corynebacterium diphtheriae* had been grown (73,74). These organisms all required added pantothenate for growth, but assimilation of pantothenate by their cells made only a small contribution to the loss. A reaction leading to inactivation of pantothenate was thus occurring and this was studied most fully in the  $\beta$ -hemolytic streptococci. The reaction was not found to occur in resting suspensions of the organisms in mixtures of inorganic salts and pantothenate. Of the constituents of the growth media, broth, yeast preparations, a casein hydrolyzate, and many

pure substances did not permit reaction with pantothenate; but reaction did occur in the presence of glucose. Its rate, about 1–4  $\text{m}\mu\text{M}$  per mg. dry weight of organisms per hour, was relatively little affected by many added substances.

The reaction did not require growth of the organisms, nor that they should remain viable, but its relationship to glucose was a relatively specific one. Inactivation proceeded anaerobically in the presence of pyruvate at only one-fourth to one-eighth the rate in the presence of glucose; it was slower if glucose were replaced by succinate or fumarate, and slower still with glycerol, citrate, 3-phosphoglycerate, yeast adenylic acid, or adenosine triphosphate. The reaction could not be induced in the absence of glucose by adding reducing agents nor by variation in pH. Various processes of extraction, including the grinding of the streptococcal cells, did not yield a cell-free preparation capable of reaction with pantothenate, either in the presence or absence of glucose.

It was found by the use of inhibitors that the circumstance necessary for inactivation of pantothenate was not only that glucose should be present, but that it should be broken down by the streptococci. The organisms produced from glucose, with or without pantothenate, about 1.9 moles of lactic acid per mole of glucose. Such glycolysis, and pantothenate breakdown, proceeded aerobically or anaerobically, but, when it was inhibited by iodoacetate, benzene, malonate, or propamidine, the reaction with pantothenate was also inhibited. The streptococcal extracts in which pantothenate was stable were also incapable of glycolysis.

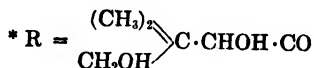
While glycolysis was proceeding at a steady rate, pantothenate inactivation was also nearly proportional to time. This was demonstrated with *Streptococcus hemolyticus*, *P. morganii*, and *C. diphtheriae*. The ratio between the two rates was relatively stable. Usually 2500 to 4500 moles of acid were produced from glucose during the inactivation of one mole of pantothenate by streptococci (71) under a variety of conditions. The ratio between the two rates was remarkably similar also, in different organisms.

**Dissociation of Glycolysis and Pantothenate Inactivation.** It is evident, however, that although pantothenate inactivation and glycolysis are closely associated in bacteria, pantothenate inactivation at the relatively large rate at which it has been reported above is not necessary to glycolysis. Streptococcal suspensions containing relatively little pantothenate are capable of rapid glycolysis and addition of pantothenate causes little change in the rate of glycolysis (65). It also had little effect on reac-

tions of *P. morganii* with glucose (see page 442). This was confirmed in experiments in which both pantothenate inactivation and rate of glycolysis were measured (71).

TABLE VI  
INHIBITION OF MICROBIAL GROWTH BY SUBSTANCES STRUCTURALLY RELATED  
TO PANTOTHENATE

Compound*	Ref. No.	Organism	Ratio (inhibitor concn./pantothenate concn.) required for inhibition of visible growth for some days
<i>dl</i> -N-Pantoyltaurine, RNHCH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> H	63	$\beta$ -Hemolytic streptococci	200
	108	<i>Streptococcus lactis</i>	8,000
	74	<i>S. faecalis</i>	15,000
	63	<i>Diplococcus pneumoniae</i>	1,000
	*74	<i>Corynebacterium diphtheriae</i>	500-10,000
	108	<i>Lactobacillus arabinosus</i>	1,000-2,000
	108	<i>L. pentosus</i>	133,000
	108	<i>Propionibacterium pentosaceum</i>	8,000
	108	<i>Leuconostoc mesenteroides</i>	133,000
	74	<i>Proteus morganii</i>	200,000
<i>dl</i> -N-Pantoyltauramide, RNHCH <sub>2</sub> CH <sub>2</sub> SO <sub>2</sub> NH <sub>2</sub>	64	$\beta$ -Hemolytic streptococci	2,000
	74	<i>D. pneumoniae</i>	10,000-50,000
	74	<i>C. diphtheriae</i>	2,000-10,000
	74		
Sulfonamide derivative, RNHCH <sub>2</sub> CH <sub>2</sub> SO <sub>2</sub> ·C <sub>6</sub> H <sub>4</sub> ·NH <sub>2</sub>		$\beta$ -Hemolytic streptococci	800
Pantamide, RNH <sub>2</sub>	74,75	" "	20,000
Pantahydrazide, RNHNH <sub>2</sub>	74,75	" "	4,000
Sulfone, (RNHCH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> SO <sub>2</sub>	74	" "	40,000
Disulfide, (RNHCH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> S <sub>2</sub>	74	" "	40,000
<i>L</i> -Phenylpantothenone, RNHCH <sub>2</sub> CH <sub>2</sub> COC <sub>6</sub> H <sub>5</sub>	129	<i>Lactobacillus casei</i>	2,500
<i>N</i> -Pantoyl-3-propanolamide, RNHCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> OH	109	<i>Leuconostoc mesenteroides</i>	300
<i>dl</i> -N-Pantoylethanolamine, RNHCH <sub>2</sub> CH <sub>2</sub> OH	109	" "	2,000
Pantoylallylamine, RNHCH <sub>2</sub> CH=CH <sub>2</sub>	107	" "	5,000
<i>dl</i> -N-Pantoyl- <i>n</i> -propylamine, RNHCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	107	" "	5,000
<i>dl</i> -N-Pantoylethylamine, RNHCH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	107	" "	10,000



Inhibitory ratios were usually determined with pantothenate concentrations of  $2 \times 10^{-8}$  to  $2 \times 10^{-7} M$ ; their variation with absolute concentrations of the reagents is small in comparison with the range of values of the present table (65). The following organisms were not inhibited by relatively high concentrations of pantoyltaurine: *Escherichia coli*, *Shigella paradysenteriae*, *Brucella abortus* (108), *Proteus vulgaris*, and *Staphylococcus aureus*. Further values are available: 75, 107, 108; see also 99.

It was observed above that many substances inhibited both glycolysis and pantothenate inactivation. Others have been found to inhibit only the reaction with pantothenate, while having little or no effect on glycolysis (73,74). All such substances known at present are derivatives of pantoic acid (Table VI) and were designed with the intention of inhibiting the action of pantothenate on bacterial growth (5,6,52,63,64,72,75,108). Of them, the most active were pantoyltaurine, an aromatic derivative of pantoyltaurine, and pantoyltauramide. *d,l*-Pantoyltaurine inhibited the inactivation of  $10^{-6}$  *M* pantothenate by  $\beta$ -hemolytic streptococci, when it was present in concentrations of about  $5 \times 10^{-6}$  *M* (Fig. 2). The inhibiting effects of these substances has been studied further in connection with their action on growth (see page 448).

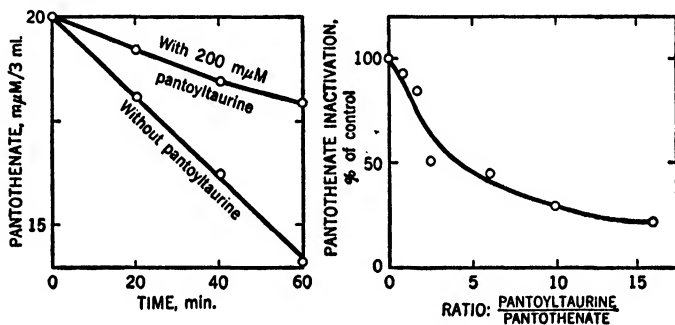


Fig. 2. Inhibition by pantoyltaurine of streptococcal inactivation of pantothenate during glycolysis (66,71).

**Rates of Pantothenate Inactivation during Bacterial Growth.** As inactivation of pantothenate at the relatively high rate of  $2 \mu M$  per mg. dry weight per hour could be dissociated from glycolysis it was relevant to investigate the extent of its connection with growth. The methods employed were those by which synthesis of pantothenate in growing cultures was measured (page 438) and they were applied to streptococcal strains and to *P. morganii* (69). The fall in pantothenate concentration, during growth of which the course was followed, was estimated and obtained as  $m\mu M$  per mg. dry weight per hour from diagrams similar to Figure 1 (page 438). Values so obtained (Table III, page 439) closely parallel the rates of reaction observed in nonproliferating suspensions. No marked changes in  $Q_p$  were associated with different phases of growth of cultures. Among the characters of the inactivation by nonproliferating suspensions,

which were retained by growing organisms, was the action of inhibitors such as pantoyltaurine (71).

#### E. INTERMEDIATE PROCESSES IN THE ACTION OF PANTOTHENATE ON GROWTH

The effect of pantothenate on microorganisms, which was first observed and in relation to which the majority of the foregoing observations were made, was its action on the over-all process of growth. In the present section further characteristics of growth are described, and it is attempted to link them with the metabolic behavior of pantothenate. Evidence obtained by the use of specific inhibitors related in structure to pantothenate has played a large part in this.

**Limitation of Growth by Pantothenate and by Pantoyltaurine.** Growth of  $\beta$ -hemolytic streptococci, *Corynebacterium diphtheriae*, and *Proteus morganii* was reduced in quantity if the pantothenate of their growth media was limited. Complete growth curves of streptococcal cultures have been obtained. With excess pantothenate, growth occurred with negligible lag, a single well-marked logarithmic phase during which the culture doubled in size each 26 to 28 minutes, and reached relatively stationary populations of about 0.5 mg. dry weight per ml. in three to four hours. Suboptimal quantities of pantothenate permitted growth after the same small lag, to commence a logarithmic phase of the usual rate of growth; but this phase was shorter than normal and the stationary population was smaller with lower quantities of pantothenate (Fig. 3).

Addition of *d,l*-pantoyltaurine (see page 446) in concentrations over fifty times those of the *l*-(+)-pantothenate present in similar culture fluids, prior to inoculation, prolonged indefinitely the lag phase of added  $\beta$ -hemolytic streptococci. If fluids of the same absolute concentration of pantoyltaurine but with pantothenate increased to more than one-tenth that of the pantoyltaurine were inoculated, normal growth ensued. Many other substances were ineffective in antagonizing the action of pantoyltaurine, and the activities of miscellaneous biological materials, such as yeast or serum preparations, could be ascribed to their content of pantothenate (63). Prolongation of lag was not accompanied by bactericidal action, nor was incubation in absence of pantothenate (65). On the basis of such observations, a simple interpretation of the action of pantoyltaurine was that it limited the availability of pantothenate to the bacteria.

Its ability to do this varied markedly in different bacterial species. Competitive relationships of the type described above, between pantoyl-

taurine and pantothenate (but without detailed examination of the course of growth) have been observed with the many organisms listed in Table VI. This records also the preponderance required of the inhibitor over pantothenate, to affect growth. Several other substances related to pantothenate inhibit growth in an analogous way (Table VI). An important characteristic of the action of pantoyltaurine, observable from such results, is that its action was confined to organisms which required added pantothenate in growth.

Closer examination of the action of pantoyltaurine on growth showed characters requiring elaboration of the simple suggestion that the substance limited the bacterial use of pantothenate. When growth was limited by lowering the concentration of pantothenate in the presence of pantoyltaurine, the phases of growth which were affected were different from those affected in the absence of pantoyltaurine (Fig. 3) (65). With pantoyltaurine, lowered pantothenate resulted in a decrease in rate of growth, and in growth with at least two logarithmic phases. The lag of the latter phase increased with increasing preponderance of pantoyltaurine but the stationary population was little affected. This latter character is the most immediately understandable, for the pantothenate concentrations employed were high enough to be changed little as a result of growth.

Other features which emphasized the need for further analysis of the relationship between pantothenate and growth were encountered in studying time relationships between the actions of pantothenate and pantoyltaurine on growth (66). Growth in the preceding experiments had been initiated by inoculating preformed mixtures of pantoyltaurine and pantothenate. If now growth was begun in the presence of pantothenate, and a large preponderance of pantoyltaurine was added during growth, no effect was seen for some time after the addition. The length of the latent period before the effects of pantoyltaurine became manifest increased with

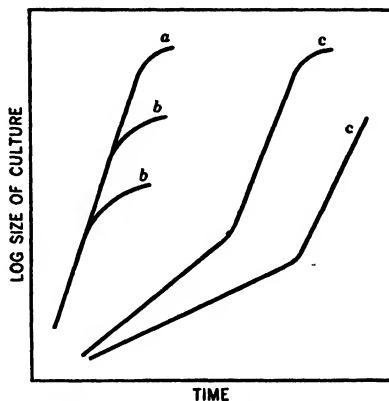


Fig. 3. Diagrammatic representation (based on 65) of the course of streptococcal growth (a) with excess pantothenate, (b) with progressively lowered concentrations of pantothenate, and (c) with excess pantothenate and with increasing concentrations of pantoyltaurine.

increasing age and size of the culture to which it was added. This is indicated in Table VII, though data are not available to differentiate between the effects of the two factors mentioned.

TABLE VII  
PROMPTNESS OF ACTION OF PANTOYLTAURINE ON STREPTOCOCCAL GROWTH (66)

Pantothenate present initially, $M \times 10^{-4}$	Pantoyltaurine added, later, $M$ <small>equiv.</small>	Population size at time of addn. of pantoyltaurine, mg. dry wt. per 3 ml. medium	Time after inoculation at which pantoyltaurine added, min.	Period of growth permitted after addn., min.	Increase in culture size permitted (ratio to size at time of addn.)
2	$10^{-3}$	0.05	15	40	1.9
2	$10^{-3}$	0.18	68	60	4.3
3.3	$3.3 \times 10^{-4}$ or $3.3 \times 10^{-3}$	0.32	170	> 75	> 5.3

Evidence bearing on the manner in which pantoyltaurine affects growth has been obtained by examining its effects in other reactions between the streptococci and pantothenate, and these effects will now be considered.

**Inhibition of Pantothenate Inactivation by Pantoyltaurine.** Inactivation of pantothenate during streptococcal growth, which can be reproduced in glycolyzing but nonproliferating suspensions (page 445), is the most rapid over-all reaction between the organisms and pantothenate. This reaction, but not the glycolysis, was strongly inhibited by pantoyltaurine (73,74). The effects of concentrations of pantoyltaurine one or two times those of the coincident pantothenate were evident in kinetic experiments of the type shown in Figure 2 (page 447). The reaction was inhibited 50% by *dl*-pantoyltaurine concentrations about five times greater than the concentrations of the *l*-pantothenate present. The process of pantothenate inactivation was thus even more sensitive to the inhibitor than was growth. Whatever its significance, it would be expected to be inhibited by some 90% during inhibition of growth by pantoyltaurine. This has been shown to be the case (71).

Further examination has shown a close relationship between inhibition of the two processes. In this, first, the action of pantoyltaurine on growth and on the inactivation in a series of organisms was compared. The concentrations of pantoyltaurine required for the inhibition varied widely in different organisms, but in parallel (74). Second, a similarly close

correlation was found when a comparison was made of the effects upon the two processes of a series of compounds related to pantoyltaurine (74).

Together with this marked parallelism between the actions of pantoyltaurine on growth and on pantothenate inactivation, it must be remembered that their sensitivities to pantoyltaurine differ, and that they differ also in a further characteristic—that is, their speed of reaction to pantoyltaurine. In experiments in which pantoyltaurine was added to suspensions of streptococci already reacting with glucose and pantothenate, and which would have shown any latent period in the action of the inhibitor of more than two minutes, no delay was detected (66). Also, if a reaction mixture in which pantothenate inactivation was already inhibited by pantoyltaurine, was replaced by one containing pantothenate without pantoyltaurine, pantothenate inactivation recommenced promptly. Pantothenate, on the other hand, did not restore pantoyltaurine-inhibited growth until after a marked interval (66). If the similarities between the two actions of pantoyltaurine indicate a functional connection between the processes affected, then the ways in which the actions differ suggest the inhibition of pantothenate inactivation to be the first process affected.

**An Inhibition of Pantothenate Assimilation by Pantoyltaurine.** The experimental arrangements in investigating pantothenate inactivation were usually such that the pantothenate of streptococcal cells constituted only a small part of the whole, and the main change discussed in the previous section has been in the pantothenate added to the suspending fluid. As, however, this change is brought about by the cells, it is relevant to inquire how pantoyltaurine is affecting any changes in the pantothenate of the organisms themselves.

Considering, first, cell suspensions in the absence of both growth and glycolysis, no change was brought about by pantoyltaurine in the quantities of pantothenate liberated to buffer solutions during some hours at 37°C. (67). Concentrations of pantoyltaurine were employed which would have inhibited completely the inactivation and, after an interval, growth. Further, if pantoyltaurine was added to glycolyzing streptococci, it had little or no effect on their concentration of pantothenate. If pantoyltaurine was added to reaction mixtures containing both glucose and pantothenate, it protected to some extent the pantothenate of the cells as well as that of the solution. In no case did it lower the pantothenate content of the cells.

The action of pantoyltaurine during growth was in marked contrast to these observations but was readily understandable in terms of them and



of other characters in its action. A concentration of pantoyltaurine which ultimately would inhibit growth completely was added to one of duplicate cultures. By virtue of the latent period in the action of the inhibitor, growth of the two cultures remained almost identical for over an hour and some three successive generations of cells could be grown in its presence. The cultures contained excess of pantothenate such that its concentration in solution did not greatly change during growth; they were thus not markedly different in pantothenate content when the cells were harvested.

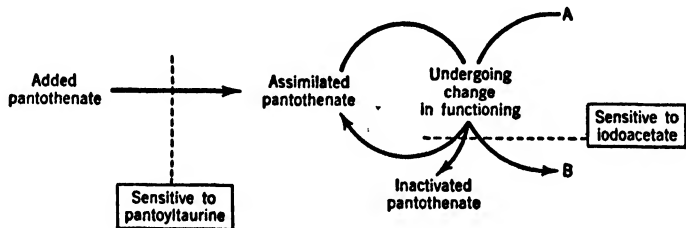
Marked differences were, however, seen in the pantothenate of the cells (67,71). In all cases, growth in the presence of pantoyltaurine yielded cells containing much less pantothenate per unit weight than did the duplicate culture. The trend of pantothenate change during growth in the control cultures was variable, in some cases increasing and in others decreasing, but pantoyltaurine always led to a further decrease. The nature of its effect could best be seen by considering the total pantothenate associated with the cells of a complete culture. Whereas this normally increased during growth, in the presence of pantoyltaurine it increased little or fell. Pantoyltaurine was thus inhibiting pantothenate assimilation, and doing so much earlier than it inhibited growth.

**Mediation of Action of Pantoyltaurine on Growth.** In attempting to place the different effects of pantoyltaurine in an order which reflects their apparent interconnection, the promptness with which they are manifest suggests the action on growth to follow those on pantothenate assimilation and inactivation. These two are inhibited rapidly. If they are not independent lines of metabolism it appears most probable that pantothenate inactivation follows assimilation, for (a) inactivation involves some interaction with the cell, the known interaction being assimilation, and (b) the products of inactivation are unknown but bring about none of the effects of pantothenate with streptococci; thus reappearance of pantothenate as the assimilated compound is not likely to follow its inactivation.

That some enzymic process in which pantothenate and pantoyltaurine compete conditions their effects on growth is suggested by analysis of the quantitative effects of partly inhibitory quantities of the substances on streptococcal growth (65). In materials examined, which included microbial extracts, pantothenate occurred as a protein derivative. The most direct interpretation of the experiments of the preceding section is that pantoyltaurine inhibits the formation from added pantothenate of a higher derivative. Action of pantoyltaurine at an early stage in the relation of pantothenate with microorganisms (rather than after its assimi-

tion) appears likely from the finding that organisms synthesizing pantothenate are not susceptible, or are relatively not very susceptible, to pantooyltaurine.

If pantothenate inactivation follows its (pantooyltaurine-susceptible) assimilation, inhibition of the inactivation by pantooyltaurine is accounted for. If assimilated pantothenate is serving some function (not susceptible to pantooyltaurine) in cells, it is reasonable that the effect of pantooyltaurine on growth should be delayed until the already assimilated substance has been "diluted" by sharing among successive generations. To go further, more knowledge is required of the reactions in which pantothenate functions, and of the extent or manner in which its inactivation is associated with them. The following scheme embodies these observations in general terms:



Reaction  $A \rightarrow B$  is associated with the reactions during which pantothenate inactivation has been observed. The performance of a reaction such as glycolysis in which added pantothenate is decomposed, does not result in decomposition of assimilated pantothenate at a comparable rate. The remainder of the molecule to which pantothenate is attached may be capable of stabilizing a limited quantity of the substance, comparable to that normally occurring in the cell.

It would appear not unreasonable to suppose that all pantothenate inactivated by streptococci has passed through the combined form found in their cells, for the quantity of this is normally about 0.1 to 1  $m\mu M$  per mg. dry weight of various organisms. Reaction at the rate of 2  $m\mu M$  per mg. dry weight per hour implies that a quantity of pantothenate equal to that in the cell is exchanged each 3 to 30 minutes. Cells not requiring added pantothenate are capable of synthesizing the substance at rates comparable to the rate of its inactivation by existing organisms, as is indeed necessary if, as is observed, their cultures are to be capable of doubling in population each 15 to 60 minutes.

#### IV. General Observations

##### A. FURTHER EXAMPLES OF METABOLIC CHANGES IN VITAMIN-LIKE SUBSTANCES

The present subject has been illustrated by changes in the two types of compound which have at present been most extensively studied. Changes in many ways comparable have been observed in several other vitamin-like substances. It is proposed to defer their detailed discussion, but the following brief account illustrates how general are the phenomena concerned, though in few cases have the metabolic changes been connected in any detail with characteristics of growth.

Thus a thiamine derivative plays a metabolic role as cocarboxylase, which has been found to be inactivated by a specific phosphatase of yeast (122,123). The inactivation was inhibited by thiamine itself and to a lesser degree by thiamine monophosphate and the pyrimidine constituent of the thiamine molecule. Synthesis and breakdown of thiamine by *Phycomyces* species have also been studied (9,45,98). Pyridoxine derivatives are now known to catalyze two types of bacterial reactions, involving transamination and decarboxylation of amino acids (4,32,35,59). Interconversion between members of the group of substances of natural occurrence which are related to pyridoxine has been observed in microorganisms and appears likely to afford a series of changes comparable to those observed in nicotinic acid derivatives. Production of folic acid from chemically defined precursors by bacterial suspensions has also been observed (110,111).

Microbial synthesis and breakdown of riboflavin (29,94,100,114) and *p*-aminobenzoate (53-55,78,79,114) have been followed. Reference has already been made to reactions in glutamine (68,70,71) and arginine (31); both these substances are essential for the growth of certain streptococci, in quantities shown to be dependent on their rates of synthesis or breakdown by the bacteria. A valuable approach has been made to defining intermediary stages in the action of biotin in yeast (128).

##### B. RELATION OF THE PRESENT SUBJECT TO OTHER BIOCHEMICAL STUDIES OF MICROORGANISMS

The present account has been especially concerned with the relations between the following three types of processes: growth, as ordinarily measured by increase in microbial substance; changes of micromolar order which afford major sources of energy and substance to the cells produced;

and changes of  $m\mu M$  order in substances which act in bringing about reactions of the preceding type.

Biochemical studies of microorganisms in general have depended to a considerable extent on experimental alteration of the balance which normally exists between these types of process. Thus the application of the resting cell technic to studies of intermediary metabolism has depended on the observation that many reactions of micromolar order continued in the absence of growth. In studies of the nature and function of growth essentials, millimicromolar processes have been restricted by restricting the quantities of millimicromolar substances presented to the cell, or by removing them, for example by washing. Such studies have shown not only that growth has been disturbed, but also that specific processes of micromolar order were affected even before effects on growth became manifest. In the present studies the course of millimicromolar processes has been observed quantitatively and compared in the presence and absence of growth. As with metabolic processes of the micromolar order, there was no certainty that millimicromolar changes would continue in the absence of growth, but many reactions of both categories have been observed to do so. It also could not be foreseen whether or not millimicromolar processes would be associated in nonproliferating suspensions with processes of micromolar order. Examples have in fact been found in which association is present, and others in which it is absent; but in general many millimicromolar processes were more firmly associated with micromolar processes than they were with the over-all process of growth.

Such findings are of general significance, for the following reason. Substances of millimicromolar order represent an important means by which the cells carry out their functions. In following associations between micromolar and millimicromolar reactions, one is following the growth or decay of subsidiary systems of the cell, which can now be seen to show a considerable degree of autonomy under certain circumstances, and yet also to be linked ordinarily with the growth of the cell as a whole. The development of such a subsidiary system of the cell may be more firmly associated with its functioning (*e.g.*, cozymase activation with yeast fermentation) than with the growth of the cell as a whole. This clearly can result in changes in the character of cells with changed substrates and so afford a mechanism of adaptation. The modes of linkage between the subsidiary systems and the cells as a whole must remain for future study, but linkage within the subsidiary system now appears reasonably accessible to biochemical investigation by technics and concepts already available.

The type of problem illustrated here by reactions of micromolar and millimicromolar orders does not appear dependent on large discrepancies between the quantities of the two substances which undergo reaction; linked reactions between glutamine and glycolysis concern quantities of the substances which differ only by a ratio of ten (68,70). The present systems approximate in some ways systems envisaged in general terms by Hinshelwood (41). However, in place of two main types of reaction postulated by Hinshelwood—a type not directly linked with growth, and another linked with growth—there has been observed a facultative linkage with growth, and a rather closer linkage with other metabolic processes. This may be due to the present study's being concerned more with the behavior of materials of coenzyme character than with the protein moiety of enzymes.

#### C. CELLULAR CONCENTRATION OF ENZYMES CAUSING MILLIMICROMOLAR REACTIONS

The following calculations (71) are relevant in considering the nature of the subsidiary systems of bacteria which are responsible for metabolism of pantothenate or nicotinic acid derivatives. The changes concerned, of about 1  $\mu$ M per mg. dry weight per hour, if in a typical organism of some  $10^{-10}$  mg. dry weight per cell, are of about  $6.4 \times 10^{23} \times 10^{-9} \times 10^{-10} \times 1/3600$ , or 18 molecules per cell per second. It has been noted above that a molecule of cozymase in its functioning in bacteria has been calculated to undergo changes with frequencies of one to five times per second, values which are possibly lowered by experimental circumstances (see page 412). A corresponding value of nearly 20 changes per second has been obtained in the case of pantothenate (Table V, page 443). These represent the rates at which some enzymes, not those concerned in pantothenate or cozymase synthesis, can react with the compounds; the routes of synthesis and inactivation are unknown but turnover numbers of some possibly relevant enzymes range from 40 to 450 moles per mole per second (34,71) at comparable temperatures. More specific knowledge of the reactions concerned is necessary but it appears feasible that the systems causing changes of the millimicromolar order should occur in bacteria to the extent of only one or a few units per cell. Their relation to the unit of inheritance is thus particularly close and presents an attractive problem. An extreme possibility consistent with the present results would be that in these instances the millimicromolar processes represent the heterocatalytic activities necessary in units of inheritance for them to influence cell processes (see

8,112). Such possibilities make the study of the reactions a matter of current interest to general biology.

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# ANTIBACTERIAL SUBSTANCES FROM FUNGI AND GREEN PLANTS

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## I. Introduction

In the eighteen years since Fleming announced the discovery of penicillin, thirty-nine antibacterial substances, including six penicillins, have been isolated in pure or nearly pure form from the thousands of fungi and green plants that have been tested. Of all these, the penicillins and streptomycin are the ones used in medicine, although others may have limited medical application. The search for medically useful substances continues in the expectation of finding a substance as good as or better than penicillin.

The concern here is primarily with the identification and the bacteriological effects of the antibacterial substances of natural origin. These are organic compounds other than the common organic acids which are formed by fungi and green plants and which are active against suitable bacteria at concentrations not greater than one milligram per milliliter of test solution. "Antibacterial" has been used instead of "antibiotic" to designate these compounds in most places because the interest is chiefly with those substances active against bacteria.

The antibacterial substances considered are those which have been well enough characterized either chemically or biologically so that uniqueness is certain. The biology of microbial antagonism has been reviewed by S. Waksman (161) and will not be discussed here. Literature citations have been kept to a minimum, with later publications given preference if they are more complete than the earlier ones or if the compound used in the earlier work was impure. The name selected for each substance is the first one applied to the pure compound or one which makes unambiguous the identity of the active substance.

The antibacterial substances are the metabolic products of fungi and green plants and are formed regardless of any "need" the plants may have for protection from invasion by or competition from bacteria. The several antibacterial substances formed by bacteria will not be considered here; the most important ones have been discussed in an earlier volume (90) of this series. The enzyme systems that form the antibacterial substances seem to be under the same type of genic control as the systems that synthesize essential metabolites and structural units. This is indicated by the results (139) of irradiating spores of *Penicillium notatum* to form strains that produce more of the penicillins than the parent strain and with ratios of the several penicillins different from that of the parent strain.

## II. Production of Antibacterial Substances

The search for new antibacterial substances is made rather systematically at present, with each group of investigators tending to specialize on small and related groups of plants. Many species and strains of plants grown in as many different environments as possible are investigated for the presence of substances active in inhibiting a test organism. Bacteria are the usual test organisms even though two substances active against fungi only are known. In a complete survey, fungi and species of bacteria representing the different groups must be used because activity against one species does not guarantee action against another unrelated species or, for that matter, even against a different strain of a susceptible species.

Members of three groups of fungi have produced most of the antibacterial substance discovered to date. Species of actinomycetes form three basic substances, one acidic substance, and actinomycin. Three acidic and two neutral substances have been isolated from the culture solution of four species of the higher basidiomycetes. The other neutral and acidic substances are formed by relatively few species of *Penicillium* and *Aspergillus*. The actinomycetes, aspergilli, and penicillia have been rather thoroughly

investigated. Few members of the phycomycetes, ascomycetes, and fungi imperfecti (142,178-180), other than penicillia and aspergilli, produce substances active against *Staphylococcus aureus*.

Three surveys, covering less than four per cent of the named species of the higher basidiomycetes, indicate that this is a good group in which to look for species that form antibacterial substances. Among 230 species of basidiomycetes found in Australia (109), sporophore extracts of 39 were active against *S. aureus* only, and 20 were active against *Escherichia coli* also.

In one survey (180) based on extracts of sporophores of 722 species in 96 genera of the higher basidiomycetes, 70 species were strongly active and 100 were weakly active against *S. aureus*, and 42 species were active against *E. coli* also. A few species were grown in liquid culture, and the culture fluid was tested, with results that frequently differed greatly from those obtained with extracts of sporophore. Tests (142) of several agar media on which 332 species and strains of basidiomycetes (mostly wood-destroying forms) had grown showed 119 species and strains active against *S. aureus*. Of these 119, 77 were active against *E. coli*. The strain of the species tested for active substance is important because all strains of one species may be active while only one of several strains of another species may produce an active substance. A negative result obtained on testing only one strain does not mean that other strains would be inactive.

The fungi are well adapted for growth on or in liquid culture media. They grow rapidly, and they have the further advantage that the active substance usually accumulates in the culture medium in much greater quantity than in the mycelium. Economical methods for handling large amounts of liquids are available.

Liquid media are used in cultures in which the fungus grows on the surface or in deep culture in which the mycelium is dispersed throughout the body of aerated solution. Surface culture technic is used for certain survey work, with fungi that will not grow in deep culture, and in laboratories that lack the rather elaborate equipment needed for the deep cultures. Considerable quantities of antibacterial substances have been produced by the surface-culture technic. The shake culture in which flasks containing layers of liquid, not too deep, are shaken continuously on a shaking machine, is a variant of the deep-culture method and is used in much of the work preliminary to deep culture. The deep-culture or the tank method is the only one that is used commercially for the production of large amounts of antibacterial substances. However, not all fungi will grow and produce active substances in the deep culture. Frequently considerable selection of media and of strains of a fungus is necessary before adequate amounts of active substance can be produced in tank cultures. The antibacterial substances formed in surface and in deep culture by the same fungus may not be the same; and if more than one active substance is formed, the ratios of the amounts of the substances may be quite different in the two types of cultures. *Aspergillus flavus* formed aspergillic acid in surface culture and a penicillin (111) in shaken cultures when grown on the same medium.

The composition of the medium influences greatly the kind and amount of antibacterial substance formed. *A. flavus* formed aspergillie acid when grown on a tryptone-brown-sugar medium and a penicillin when grown on a modified Czapek-Dox medium in surface culture (111). The production of penicillin by *Penicillium notatum* could be greatly affected by changing the total concentration of salts (131). For maximum yield of penicillin, the proper balance of concentrations among phosphate, sulfate, nitrate, and magnesium ions must be obtained (131).

Different antibacterial substances are formed at different phases of the growth of several fungi; one substance may be formed early and another one after a considerable period. Kojic acid is formed during the first 12 days of growth of *Aspergillus parasiticus* (55) but disappears rapidly. About the twenty-fourth day a penicillin-like substance is produced and persists for as long as 55 days. The antibacterial activity of a culture solution of *A. fumigatus* (80) is due to gliotoxin at the end of four days but to helvolic acid after two or three weeks.

One strain of a species of fungus may produce an antibacterial substance abundantly under one set of conditions and be quite useless under another set. Other strains may be completely useless. An enormous amount of work has been expended in producing, isolating, and testing strains of *P. notatum* (139,140). Sufficiently detailed investigation has revealed that high- and low-yielding strains can be isolated from many fungi. Certain strains of *Trichoderma viride* form gliotoxin, some form viridin, and others do not produce any substance active against bacteria or fungi (27).

Eight antibacterial substances have been isolated from higher plants. One of these, citrinin, was first isolated from the culture solution of a fungus. In a survey (117) of the flowering plants, 2300 species belonging to 166 families were tested; and 68 genera, belonging to 28 families had species active against *S. aureus*. Extracts of 450 types (11) of flowering plants, mostly native to Australia, tested by the cylinder-plate method, showed 38 active against *S. aureus* but not active against *Eberthella typhosa* and only one active against both bacteria. Active substances, several volatile with steam, were found in the flowers and, sometimes, in the leaves also. Members of 23 genera in 15 families of common higher plants were investigated for strains that formed substances active against *S. aureus*, *E. coli*, and *Bricella abortus* (91). Active substances were produced by members of six genera belonging to six families.

#### METHODS OF ISOLATION

The naturally occurring antibacterial substances are composed of various combinations of carbon, hydrogen, nitrogen, oxygen, sulfur, and chlo-

rine. About half of them are composed of the elements, carbon, hydrogen, and oxygen, only. The substances can be put into one of the four groups of compounds: acidic, neutral, basic, or proteinaceous. The compounds in the acidic group are the carboxylic acids and acidic substances that are acid enough to be extracted from aqueous solution at low pH by organic solvents immiscible with water. The neutral compounds are without appreciable acidic or basic properties. The basic compounds contain basic nitrogen groups and are precipitated by base precipitants. The chemical nature of the antibacterial substance determines the method of isolation.

The object of the chemical separations is the preparation of the active substance in a pure and crystalline form, if it is a solid, for use in chemical and biological investigations. The chemist wants to determine the structure and to synthesize it. The biologist wishes to ascertain its biological properties. Both want to work with pure crystalline material to avoid the possibility that the properties are modified or determined by impurities in the noncrystalline preparations.

Crystalline form is not a guarantee of purity. The distinction between chemical and biological purity should always be kept in mind. A product that is pure enough for most chemical work could be grossly contaminated with respect to its biological activity. For example, any other antibacterial substance now known that contained 1% of chaetomin or 4% of penicillin as impurity would have an action on many bacteria due wholly or appreciably to the impurity and yet be "chemically pure" for many purposes.

The four general methods used for preparing concentrates of antibacterial compounds are: extraction with organic solvent; adsorption on a suitable solid, usually activated carbon; concentration of the culture solution by low-temperature evaporation; and precipitation. The last two methods are little used directly on culture solutions but are more often applied to concentrates prepared by the first two methods. Many of the antibacterial substances are separated from most of the materials in the culture liquid or from ground plants by extraction with an organic solvent under suitable conditions. Several substances, such as streptomycin (145), streptothricin (160), and the active material occurring in *Crepis taraxacifolia* (85) are concentrated by adsorption on activated carbon and eluted from it by suitable solutions. The acidic substances can be extracted from acidified aqueous solution by an organic solvent and then recovered from the solvent by extracting it with a solution of sodium bicarbonate or with a buffer solution. This simple procedure often effects a concentration of one hundred times and separates the acidic substances from most of the other compounds in the culture filtrate. The concentrate can be worked up in several ways depending upon the properties of the active substance and the accompanying impurities. If the extraction of the organic solvent by bicarbonate is followed by extraction with a solution of sodium carbonate or sodium hydroxide, weakly acidic substances are removed; and they can be processed in much the same way as the more strongly acidic substances. The neutral

compounds remaining in the organic solvent usually can be recovered by evaporation of the solvent. If the presence of more than one substance soluble in the organic solvent is suspected, the number can be ascertained and, perhaps, they can be isolated by applying the countercurrent distribution method (87) to the concentrates.

### III. Chemical and Biological Properties of Antibacterial Substances

The descriptions of the chemical and biological properties of the antibacterial substances of natural origin are scattered through medical, chemical, and biological journals, many of which are not readily available. As the ever-growing list of antibacterial substances lengthens, the establishment of the lack of identity of each new one with all of the previously described ones becomes more difficult. To add to the confusion, the same substance has been described under several different names; and a pure product and a preparation of it contaminated with large amounts of a quite different and more active impurity have been given the same name.

The antibacterial compounds are grouped into acidic, neutral, or basic substances and the chemical and biological properties are given for each. The protein, notatin, is also included. As much of the following information as is known is listed for each substance: name, empirical formula, structural formula, melting point, color, crystal form, optical activity, adsorption bands, solubility, special chemical properties, derivatives that might be useful in characterization, stability, organisms forming it, media, yield, occurrence intra- or extracellularly, general antibacterial properties, and toxicity. Some of the chemical and physical properties are summarized in Table III on page 486. The discussion of the chemistry of the antibacterial substances will be kept to the minimum needed for isolation and identification of the active principles since publication of the details of the chemistry of the penicillins and streptomycin can be expected soon. Oxford (120) has summarized the structural chemistry of actinomycin, citrinin, helvolic acid, patulin, penicillic acid, and puberulic acid. The unnamed antibacterial substances from green plants are listed under the scientific name of the plant, with the understanding that it is the antibacterial substance and not the plant that has the properties.

**Actinomycin.** Actinomycin A (164,170),  $C_{38-41}H_{49-56}N_7-8O_{9-11}$ , m.p. 250°C., red platelets,  $[\alpha]_D^{25} = -320^\circ$ , absorption peaks at 2400 and 4500 Å; soluble in acetone, alcohol, benzene, 10% hydrochloric acid; slightly soluble in water and ether; insoluble in dilute acids, dilute alkalis, and petroleum ether. It is readily reduced by sodium hydrosulfite and by stannous chloride but not by sodium bisulfite. Several groups can be acetylated. It seems to be a quinone containing free hydroxyl groups. Oxford (120) thinks that it may not be a true para quinone and that it might contain a polypeptide chain, like gramicidin, as well as a chromo-

phoric group. Actinomycin can be boiled for thirty minutes in dilute alcoholic solution without destruction but boiling in dilute acid or dilute alkali destroys it. It is formed by *Actinomyces antibioticus* grown on a starch-tryptone medium. The yield is about 100 mg. per liter of culture filtrate. It is active against Gram-positive bacteria only. It kills mice when given intraperitoneally or subcutaneously at 500  $\mu$ g. per kg., or orally.

**Allium sativum.** The compound,  $C_6H_{10}OS_2(C_6H_5SOSC_6H_5)$  (41,47), a colorless oil,  $d_{20}$  1.112,  $n_D^{20}$  1.651, optically inactive, only end absorption in ultraviolet, neutral substance, soluble in alcohol, benzene, ether, water (25 mg. per ml. at 10°C.), and nearly insoluble in petroleum ether. The oil is immediately decomposed by alkali with precipitation of allyl disulfide and formation of alkali sulfite. It cannot be dry-distilled without destruction. A solution of potassium permanganate and bromine water are rapidly decolorized. It reacts with cysteine at pH 6 to form *S*-(thioallyl)-cysteine in quantitative amounts. Cysteine and *N*-acetylcysteine but not *S*-methyleysteine rapidly destroy its antibacterial activity. Dilute acids are without effect. There is rapid loss of activity when solutions are heated. About 1.5 g. of oil can be obtained from 1 kg. of fresh cloves of *Allium sativum*. The oil exists in the plant in a stable and odorless combined form from which it is released by enzymic action (43). It has an LD<sub>50</sub> of 60 mg. per kg. body weight when given to mice intravenously. (LD<sub>50</sub> is the dose that kills 50% of the animals in a given length of time.) The oil is equally active against Gram-positive and Gram-negative bacteria.

**Arctium minus.** This compound (45),  $C_{15}H_{20}O_5$ , m.p. 115–117°C. (dec.), colorless prisms,  $[\alpha]_D^{25} = +120^\circ$  in acetone, only end absorption in ultraviolet, neutral substance; soluble in acetone, alcohols, chloroform, dioxane, and ethyl acetate; slightly soluble in ether, benzene, and water (0.2%, 25°C.); insoluble in petroleum ether. Its molecule contains a potential carboxyl group (possibly as a lactone) no methoxyl, and one double bond. The isopropylamine derivative melts at 174°C. A dilute aqueous solution lost no antibacterial activity on standing 20 days at 25°C. Alkalies cause rapid inactivation. Dilute acids were without effect on activity. There was a slight loss on heating the aqueous solution at 100°C. for 15 minutes or at 60°C. for 24 hours. It is inactivated by cysteine and *N*-acetylcysteine but not by *S*-methyleysteine. It was isolated from leaves of *Arctium minus* in quantities of from 3–18 g. per kg. dry leaves. Fresh or dried leaves could be used. It has low activity against Gram-positive bacteria only. The LD<sub>50</sub> = 90 mg. per kg. body weight when given intra-

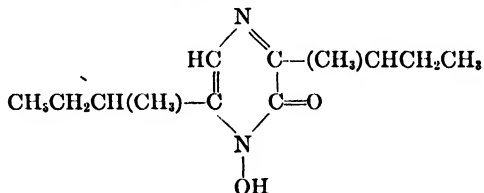


venously to mice. It did not protect mice infected with *Streptococcus hemolyticus* C 203.

**Asarum canadense.** Product A (42),  $C_{21}H_{20}O_8N_2S(?)$ , decomposes above  $160^\circ C.$ , colorless, single absorption peak at  $2820 \text{ \AA}$ , neutral substance; soluble in ethanol, acetone, chloroform, ethyl acetate, and dioxane; almost insoluble in water, benzene, and petroleum ether. Treatment with alkali ( $pH$  10) liberated acidic groups with the formation of sulfide ion and a substance with greenish fluorescence and destroyed the antibacterial activity. Stability at other  $pH$  values was not given. It is inactivated by cysteine. Product A is found in the leaves and stems of *Asarum canadense* var. *reflexum*. About 20 mg. can be obtained from each kilogram of fresh leaves and stems. It is active against Gram-positive bacteria only. Toxicity was not given.

**Asarum canadense.** Product B (42),  $C_{16}H_{11}O_7N$ , does not melt, darkens slowly  $>230^\circ C.$ , lemon-yellow needles, absorption peaks at 2500, 3180,  $3900 \text{ \AA}$ , acidic substance; soluble in ethanol, acetone, chloroform, ethyl acetate, and dioxane; almost insoluble in water, benzene, and petroleum ether. The salts are soluble in water. Product B is stable at  $pH$  10. It is inactivated by cysteine. It was isolated from the leaves and stems of *Asarum canadense* var. *reflexum* (wild ginger) in a yield of about 20 mg. per kg. of fresh leaves and stems. It has low activity against Gram-positive bacteria only. Toxicity was not determined.

**Aspergillic Acid.**  $C_{12}H_{20}N_2O_2$ . Structure (62):



Bright yellow prisms, m.p.  $93^\circ C.$ , structure not proved by synthesis; soluble in alcohol, benzene, isopropyl acetate, acetone, and 2% sodium bicarbonate solution; nearly insoluble (0.07 mg. per ml.) in water and acids. Aspergillic acid has a  $pK_a$  of 5.5. The hydrochloric acid derivative melts at  $178^\circ C.$  It forms a grass-green cupric salt with m.p.  $198^\circ C.$  (62). Aspergillic acid is remarkably stable toward acidic and alkaline hydrolytic agents (62). Selected strains of *Aspergillus flavus* Link (177) produce as much as 400 mg. of crystalline aspergillic acid per liter of culture fluid (96) when grown on a tryptone-brown sugar medium. Aspergillic acid is ac-

tive against Gram-positive and Gram-negative bacteria, mycobacteria, and fungi (35,96). It has an LD<sub>50</sub> of 48.5 mg. per kg. body weight when given to mice intravenously (137).

**Aspergillin** (Stanley). See Gliotoxin.

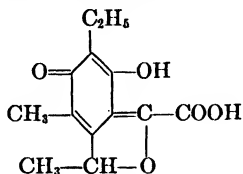
**Biformin**. This compound (142b) is a neutral substance; soluble in alcohol, chloroform, ether, and methyl isobutyl ketone; moderately soluble in water, not volatile with steam, precipitates with silver nitrate, does not liberate iodine from acidified potassium iodide solution, or react with potassium cyanide, or give a color with ferric chloride solution, thermally stable at pH 3-8. Biformin solutions cannot be evaporated to dryness, even in high vacuum, without causing destruction of the active substance, presumably by polymerization. Biformin is a highly unsaturated, reactive compound. *Polyporus biformis* grown on a Czapek-Dox corn-steep-liquor medium forms about 80 mg. per liter. It occurs in the culture free and in combined (inactive) form from which it is released by heating the solution to 100°C. Biformin is active against Gram-positive and Gram-negative bacteria, mycobacteria, and fungi.

**Cassic Acid**. This compound (142c) forms yellow microcrystalline needles, dec. 342°C., absorption peaks at 2700 and 4250 Å, not very soluble in organic solvents and water, forms a soluble sodium salt. Cassic acid gives the purplish-red color in alkaline solution characteristic of such anthraquinone derivatives as "emodin" (4,5,7-trihydroxy-2-methylanthraquinone) and "rhein." Cassic acid does not release iodine from hydriodic acid or give a color with ferric chloride or potassium cyanide. It is stable for at least several months in alkaline (pH 8-9) solution at 11°C., and thermostable in neutral or slightly alkaline solution. It is isolated from the leaves of *Cassia reticulata* in yields of about 4-5 g. per kg. of dry leaves. It is active against Gram-positive bacteria. It is not very toxic toward white mice.

**Chaetomin**. Chaetomin (79,162), CHONS (empirical formula unknown), not crystalline, m.p. 218-220°C. dec., neutral substance; soluble in acetone, ethyl acetate, chloroform, and benzene; less soluble in ether and alcohol; insoluble in water and petroleum ether. Chaetomin can be heated in water to 100°C. for ten minutes. It is stable in alcoholic solution and in 0.01 N hydrochloric acid at room temperature for 24 hours. It is destroyed in 24 hours at room temperature by 5% sodium carbonate solution. Chaetomin is produced by *Chaetomium cochliodes* grown on Czapek-Dox and corn-steep media in static and shaken cultures. Most (95%) of the chaetomin is found in the mycelium. A liter of culture fluid may yield

from 30–75 mg. crude chaetomin. Chaetomin is active only against members of the Gram-positive group of bacteria. Chaetomin is not toxic to mice but does not protect them against infection with Gram-positive bacteria.

**Citrinin.**  $C_{13}H_{14}O_5$ . Structure (86):



m.p.  $168^{\circ}\text{C}$ ., yellow microscopic needles or plates, structure not proved by synthesis and is in doubt (82,153), has properties of a phenol and of a carboxylic acid; soluble in alcohol, dioxane, bicarbonate solutions; insoluble in water. Ferric chloride forms at first a heavy buff-colored precipitate which dissolves in excess of reagent to give an intense iodine-brown solution. Citrinin also gives a deep red color when its solution is treated with hydrogen peroxide and then made alkaline with sodium hydroxide (156). It is stable and resists autoclaving (158). Citrinin is formed by *Penicillium citrinum* Thom (133) grown on Czapek-Dox and Raulin-Thom media, by *Aspergillus* spp. (157) grown on Czapek-Dox, and by the flowering plant, *Crotalaria crispata* (64). Citrinin inhibits the growth of the Gram-positive bacteria only. It is lethal to mice at 100 mg. per kg. body weight when given intravenously (158).

**Clavacin, Clavatin, and Claviformin.** See Patulin.

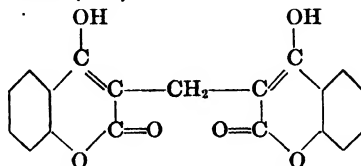
**Crepis.** The compound (85),  $C_{14}H_{16}O_4 \cdot H_2O$ , or  $C_{15}H_{18}O_4$ , no melting point, darkens above  $300^{\circ}\text{C}$ ., neutral substance, soluble in acetone, very soluble in pyridine, soluble 0.25 mg. per ml. water and 2.5 mg. per ml. alcohol, adsorbed on charcoal and eluted with 80% acetone, no carboxyl or methoxy group, one ethyl, and one or two hydroxyl groups per molecule. There is evidence for a  $\beta, \gamma$  unsaturated lactone. Activity is destroyed by 0.1 *N* alkali in thirty minutes and one acidic group is revealed. It decolorizes a solution of permanganate, adds four bromine atoms, or absorbed four hydrogen atoms per molecule. It did not reduce Fehling solution or ammoniacal silver nitrate. It has no ketone or aldehyde groups. It is stable in acid solution and could be autoclaved in 0.1 *N* hydrochloric acid at  $120^{\circ}\text{C}$ . for twenty minutes without losing activity. It is slowly destroyed at pH 9. About 100 mg. of crystals is obtained from 1 kg. of fresh flowers and buds of *Crepis taraxacifolia* (Thuill.). Activity on bacteria is slight.

The titer was decreased about half for a hundred-fold increase in concentration of bacteria in the test. Semiquantitative antibacterial activity in gramma per milliliter is given below:

<i>Staphylococcus aureus</i> .....	33
<i>Streptococcus pyogenes</i> .....	132
<i>Bacillus subtilis</i> .....	66
<i>Salmonella typhi</i> .....	250

Toxicity was not determined for animals.

**Dicoumarol.** This compound (37) has the empirical formula,  $C_{19}H_{12}O_6$ , and its structure (154) is:



3,3'-methylenebis(4-hydroxycoumarin)

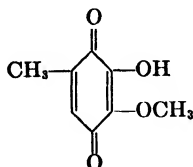
Synthesized, m.p. 288–9°C., colorless hexagonal prisms, optically inactive, absorption peaks at 2880, 3085, 3235 Å. slightly soluble in benzene; less soluble in acetone, ether, and cyclohexanone (8 mg. per ml.); insoluble in alcohols, ketones, hydrocarbons, and water, 1 mg. per ml. soluble in dilute sodium carbonate or sodium hydroxide, no reducing action in warm alkaline solutions. Dicoumarol forms a dimethyl ether, m.p. 168–170°C., and a diacetate, m.p. 250°C. dec. Its acidity is due to two enol structures. Mild alkali opens the lactone rings. Dicoumarol is formed by the spoilage of coumarin-containing sweet clover and causes the hemorrhagic sweet clover disease of cattle. It is active against Gram-positive bacteria and its action is not antagonized by 2-methyl-1,4-naphthoquinone, a physiological antagonist of dicoumarol (83).

**Expansine.** See Patulin.

**Flavicipidin and Flavicin.** See Penicillin.

**Fumigacin.** See Helvolic Acid.

**Fumigatin.** This compound (6) has the empirical formula,  $C_8H_8O_4$ , and the structure:

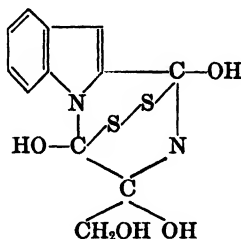


3-hydroxy-4-methoxy-2,5-toluquinone

Synthesized (14), m.p.  $116^{\circ}\text{C}$ ., maroon-colored needles, acidic substance, soluble in acetone, chloroform, benzene, ether, ethanol, and ethyl acetate; fairly soluble in water and in boiling petroleum ether. Fumigatin gives an intense purplish black color in alcoholic solution with ferric chloride. Solutions in sodium bicarbonate and sodium hydroxide have a permanganate color. It cannot be heated in aqueous solution at  $45^{\circ}\text{C}$ . for any length of time without decomposing partially. Dilute aqueous solutions decompose at room temperature in diffuse daylight. Fumigatin is formed by *Aspergillus fumigatus* Fresenius grown on Czapek-Dox medium. About 40 mg. of fumigatin is formed in each liter of culture fluid. Fumigatin is active mainly upon Gram-positive bacteria. Toxicity for animals is unknown.

**Gladiolic Acid.** This compound (26),  $\text{C}_{11}\text{H}_{10}\text{O}_5$ , a methoxymethyl-2-carboxyphenylglyoxal, m.p.  $160^{\circ}\text{C}$ ., long colorless silky needles, optically inactive, monobasic acid, soluble in ether and water, adsorbed by activated carbon and eluted from the dried carbon by ether. Gladiolic acid gives a deep green color with concentrated ammonia; the color changes to red after twelve hours. It is stable for at least ten days at  $25^{\circ}\text{C}$ . and pH 3-8. It is formed by *Penicillium gladioli* McCull and Thom grown on a Raulin-Thom medium containing 7.5% dextrose. The cultures are harvested when the activity indicates 64 mg. of gladiolic acid per liter of solutions. It is active against Gram-positive bacteria and fungi. In nutrient broth at pH 7, *Staphylococcus aureus* is inhibited at a concentration of 250 micrograms per ml., and *Escherichia coli* and *Salmonella typhi* are not inhibited at twice that concentration. Spores of *Botrytis allii* are inhibited at 2  $\mu\text{g}$ . per ml. (pH 3.5).

**Gliotoxin.** This substance (174),  $\text{C}_{13}\text{H}_{14}\text{N}_2\text{O}_4\text{S}_2$  (94), has the structure (61):



Instantaneous m.p.  $221^{\circ}\text{C}$ . (dec.) (in a slowly heated bath, decomposition begins at  $165^{\circ}\text{C}$ .), not synthesized, white needles (175) or platelets (94),

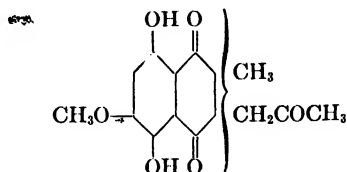
$[\alpha]_D^{25} = -290^\circ$  in ethanol, broad absorption band with peak at 2700 Å (94), neutral substance; soluble in chloroform, alcohol, dioxane, ethyl acetate, and pyridine; slightly soluble in dilute acids, and nearly insoluble in water (0.07 mg. per ml. at 30°C.). Gliotoxin is stable in acid solution and is rapidly inactivated by alkali or even by a sodium bicarbonate solution. Heating at 100°C. for five minutes in 1% sodium bicarbonate solution destroys gliotoxin completely (113). It is reversibly inactivated by thiols (46). Permanganate is reduced (33). Gliotoxin is produced by *Aspergillus fumigatus* mut. *helvola* (Yuill.) (80), *A. fumigatus* (113,155), *Penicillium jenseni* (29), *P. obscurum* Biourge (116), and *Trichoderma viride* (25,28,173,175). *T. viride* forms about 75 mg. of gliotoxin per liter of an acidified Weindling's medium (172) and can be grown in static, shake, or drip culture (28). Gliotoxin has general antibacterial activity and is active against many fungi including plant and animal parasites. For mice the LD<sub>50</sub> is 19.5 mg. per kg. by the intravenous route (137).

**Glutinosin.** (31). Empirical formula C<sub>48</sub>H<sub>80</sub>O<sub>16</sub> (?), m.p. >300°C., colorless platelets,  $[\alpha]_D^{20} = +54^\circ$ ; soluble in ether, *n*-butanol, benzene, and petroleum ether. It is stable in aqueous solution in the pH range 2.9 to 7.6. It is formed by *Metarrhizium glutinosum* grown on Raulin-Thom medium supplemented by 0.01% yeast extract. Yields of the order of 15 mg. per liter of culture filtrate were obtained. It does not inhibit *Staphylococcus aureus* or *Escherichia coli* at 100 µg. per ml. It does prevent germination of fungus spores. A concentration of 0.2 µg. per ml. prevents germination of spores of *Botrytis allii*. Glutinosin has antifungal properties resembling those of viridin but is a more stable substance.

**Helvolic Acid.** Empirical formulas given as C<sub>232</sub>H<sub>44</sub>O<sub>8</sub> (50) and C<sub>238</sub>H<sub>228-43</sub>O<sub>7-8</sub> (113), m.p. 212°C. (micro), 204–209°C. (cap. tube) (50), 215–220°C. (113), white needles,  $[\alpha]_D^{25} = -132^\circ$  in chloroform (113), acidic substance; soluble in acetone, acetic acid, chloroform, and pyridine; moderately soluble in benzene and ether; slightly soluble in petroleum ether; and insoluble in water. The sodium salt is soluble in water. Titration indicates one carboxyl group and one masked acidic group. The molecule contains one acetyl group. It forms a silver salt. The methyl ester has a m.p. 260–261°C., the oxime, m.p. 204–206°C. (113). Helvolic acid does not reduce Fehling solution immediately or ammoniacal silver nitrate. It is not affected by boiling for ten minutes in 2 *N* acid or at pH 7 or 10, but is destroyed by boiling in 1 *N* sodium hydroxide (50). It reacts rapidly with bromine and with permanganate in sodium carbonate solution (113). Helvolic acid is formed by *Aspergillus fumigatus* mut. *helvola*, Yuill. growing

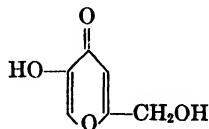
on Czapek-Dox medium (50) and by *A. fumigatus* grown on Czapek-Dox medium (165,166). From 7 to 18 mg. of crude acid can be obtained from one liter of culture fluid (113,163). Helvolic acid inhibits Gram-positive bacteria primarily. For mice injected intraperitoneally, the  $LD_{50} = 400$  mg. per kg. It did not protect mice infected with *Streptococcus hemolyticus* C 203, although life was prolonged (113). Repeated doses caused damage to the liver (50).

**Javanicin.** This compound (10) has the formula  $C_{15}H_{14}O_6$ :



red laths, m.p.  $208^{\circ}\text{C}$ . (dec.), soluble in ether and benzene, nearly insoluble in petroleum ether, extracted from organic solvents by sodium carbonate solution. It forms violet-colored alkaline solutions. It acts as a weak, monobasic noncarboxylic acid. It forms a yellow anhydromonoacetyl derivative, m.p.  $247\text{--}248^{\circ}\text{C}$ . Javanicin is accompanied by a second pigment "oxyjavanicin," in which the ring methyl group is replaced by a hydroxy methyl group. Oxyjavanicin,  $C_{16}H_{14}O_7$ , melts at  $212^{\circ}\text{C}$ . About 100 mg. of crystals can be isolated from a liter of glucose-tryptone medium on which *Fusarium javanicin* has grown. The antibacterial action is against Gram-positive bacteria and mycobacteria. *Staphylococcus aureus* and *Mycobacterium phlei* were inhibited at  $2.5\text{ }\mu\text{g}$ . per ml. *Escherichia coli*, *Streptococcus pyogenes*, and *Bacillus pyocyaneus* are inhibited slightly or not at all. It was not very toxic toward mice.

**Kojic Acid.**  $C_6H_6O_4$ :



m.p.  $153^{\circ}\text{C}$ ., colorless prismatic crystals, acidic substance; soluble in water, alcohol, and ethyl acetate; less soluble in ether, chloroform, and pyridine. The hydroxyl group attached to the ring is responsible for the weak acidic properties. Kojic acid forms a cupric salt but is not precipitated by mercuric ion, lead acetate, or silver nitrate. It reduces Fehling solution and

gives a quantitative iodoform reaction (15). Yields of 8–9 g. per liter were obtained from *Aspergillus effusus* Tiraboschi grown on a maltose–malt extract–peptone medium (93). However yields of 70–100 g. per liter were obtained when *A. luteo-virescens* was grown on Raulin-Thom medium containing 400 g. dextrose per liter (115). It is also formed by the *A. oryzae-flavus* group, *Penicillium daleae* (15), and *A. parasiticus* (55). It has low activity against Gram-positive and Gram-negative bacteria. It is toxic for mice at 200 mg. per kg. by either the intravenous or the intraperitoneal routes (93). The LD<sub>50</sub> for mice was 120–180 mg. per kg. intraperitoneally (115).

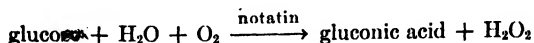
**Litmocidin.** M.p. 141–146°C. (dec.), red powder, acidic substance; soluble in ethanol, acetone, ether, and amyl acetate; nearly insoluble in water; forms water-soluble salts. Its solutions are red in acid, violet in neutral, and blue in alkaline solution (hence the name). It is adsorbed on activated carbon and is eluted by acid acetone. It is stable to boiling in acid solution but is destroyed under the same conditions in alkaline solution. The pigment may be related to the anthocyanidines (24). Litmocidin is formed when a nonsporulating strain of *Proactinomyces cyaneus-antibioticus* is grown on tryptone–peptone–glucose agar for two days at 28°C. The concentration of active substance was about 500 mg. per liter of medium at the end of two days of growth. It is active against Gram-positive bacteria and nearly inactive against Gram-negative bacteria. Its action is not affected by serum. Large doses did not protect mice from infection with *Staphylococcus aureus*. The LD<sub>50</sub> = 50 mg. per kg. and LD<sub>100</sub> = 125 mg. per kg. when given intraperitoneally to mice (77).

**Mycophenolic Acid.** C<sub>17</sub>H<sub>20</sub>O<sub>6</sub>, m.p. 140°C., colorless needles (5), acidic substance; soluble in alcohol, ether, and chloroform; very soluble in hot toluene; almost insoluble in water. It gives a violet color with ferric chloride in aqueous solution and a bright green color in alcoholic solution. It is a monocarboxylic and probably a dibasic acid, forms an insoluble barium salt, and has no reducing action (5). It was formed by *Penicillium stoloniferum* Thom growing on Raulin medium and was isolated from the mycelium (5) and by *P. brevi-compactum* Dierckx. (NCTC 3568) (1). Mycophenolic acid is most active against *S. aureus*, but the bacteria adapt rapidly and grow in the presence of the acid (1). It kills all mice when given intravenously at a level of 500 mg. per kg. (70).

**Notatin.** Notatin (56), also known as penicillin A, penicillin B (22), and penatin (104). Flavoprotein enzyme, buff-colored powder, absorption maxima at 2800, 3750, 4650 Å (56); soluble in water, insoluble in organic

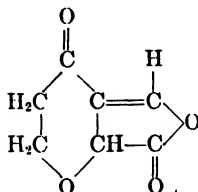


solvents; fairly stable at  $pH$  2-7. It is inactivated at  $pH$  values below 2 and above 8, by heating in aqueous solution to  $60^{\circ}C.$ , by 70% methanol at  $30^{\circ}C.$ , and by trichloroacetic acid (57,159). Notatin is formed by *Penicillium notatum* (57) and *P. resticulosum* (57) grown on a Czapek-Dox medium in surface culture. One liter of culture fluid yields 30-40 mg. of notatin. The antibacterial action of notatin is due to the hydrogen peroxide formed from the sugar of the medium (57). The reaction may be represented by:



Notatin is not very toxic to animals.

**Patulin.** Patulin (9), also known as clavacin (165), clavatin (18), claviformin (49), expansine (108),  $C_7H_6O_4$ :

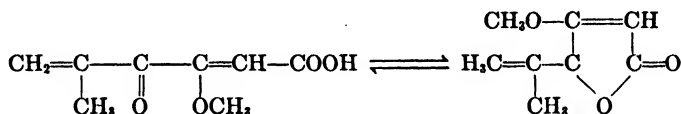


Anhydro-3-hydroxymethylenetetrahydro- $\gamma$ -pyrone-2-carboxylic acid, not synthesized, m.p.  $109-111^{\circ}C.$  (dec.), large colorless tablets, optically inactive; soluble in water, ethyl acetate, alcohol, and acetone; moderately soluble in ether and chloroform; insoluble in benzene and petroleum ether (49). Patulin reduces Fehling solution in the cold. It does not give a color with ferric chloride. On treatment with cold dilute alkali nearly two equivalents of acid are unmasked and the solution now gives a strong red color with ferric chloride (132). It forms a monoacetyl derivative, m.p.  $118-120^{\circ}C.$ , an oxime, m.p.  $152-155^{\circ}C.$ , and a phenylhydrazone, m.p.  $149-150^{\circ}C.$  (18). The antibacterial activity is destroyed by treatment with excess alkali. The compound hydrolyzes to the inactive acid on standing in aqueous solution at room temperature for fourteen days (132). Alcoholic solutions (100) and solutions in  $pH$  6 phosphate are stable (132) for three months. Patulin can be boiled (49) in 0.1  $N$  hydrochloric acid for thirty minutes without destruction, but boiling at  $pH$  7 for ten minutes destroys most of its activity. The antibiotic activity of patulin is inactivated by sulfhydryl compounds (40). Patulin is formed by *Penicillium patulum* Bainier (9), *P. expansum* (Link) Thom (9,103), *P. claviforme* (18,49), *P. urticae* Bain. NCTC 1215 (103), *Aspergillus clavatus* (18,89,

100), *A. terreus* Thom NCTC 981 (103), *Gymnoascus* sp. (98). The fungi can be grown on Czapek-Dox, Raulin-Thom, 2% malt, or potato-dextrose media. About one gram per liter of crystalline patulin was isolated from a Raulin-Thom tartrate medium on which *P. patulum* had grown for twelve days at 24°C. (132). Patulin is active against Gram-positive and Gram-negative bacteria, fungi, and mycobacteria. Patulin is very toxic to plant and animal cells. A concentration of 1.25 µg. per ml. killed all leucocytes in one hour (49). For mice the LD<sub>50</sub> was 20 mg. per kg. by intravenous and subcutaneous routes (100,132). Edema results from damage to capillaries. Patulin relaxes smooth muscles (32).

**Penatin.** See Notatin.

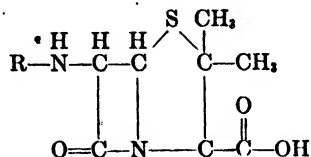
**Penicillic Acid.** C<sub>8</sub>H<sub>10</sub>O<sub>4</sub> (124):



$K_a = 1.26 \cdot 10^{-6}$ , 25°C. (aqueous solution), anhydrous acid, m.p. 86°C., pale yellow crystals of the dihydrate crystallize from hot water (m.p. 64–65°C.); very soluble in chloroform; soluble in water (2 g. per 100 ml.), alcohol, ether, and benzene; insoluble in petroleum ether. It reduces cold ammoniacal silver nitrate but not Fehling solution. Dilute ammonium hydroxide slowly converts it to a red dye (5). Its antibacterial action is inhibited by sulfhydryl compounds (78). Penicillic acid is formed by *Penicillium puberulum* Bainer grown on Raulin medium (5), by *P. cyclopium* (122), by *P. Thomii*, by *P. suavolens*, and by *Aspergillus ochraceos* (99). Penicillic acid is active against Gram-positive and Gram-negative bacteria. It is toxic to mice when given subcutaneously at 300 mg. per kg. (5).

**Penicillin A and Penicillin B.** See Notatin.


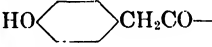
**Penicillins.** The penicillins are a group of at least six closely related organic acids with the general formula:



The chemistry of the penicillins has not been published and the structures proposed (182) are not accepted by all of the chemists who have worked on

the problem. The literature on penicillin is extensive and only the minimum necessary for the purposes of this review will be cited. Table I gives the structures of the penicillins that have been studied. The penicillins have a  $pK$  value of 2.8 (182). The sodium salt of penicillin G is  $C_{16}H_{17}O_4 \cdot N_2SNa$  with a molecular weight of 358.

TABLE I  
STRUCTURE OF THE PENICILLINS (CHARACTERISTIC "R")

Designation		Characteristic "R"		Produced (138a) by
American	English	Name	Structure	
F	I	3-Hexenoic	$CH_3CH_2CH=CHCH_2CO-$	<i>P. notatum</i>
Flavicipin		4-Hexenoic	$CH_3CH=CHCH_2CH_2CO-$	<i>A. flavus</i>
Dihydro F		Caproic	$C_6H_{11}CO-$	<i>A. giganteus</i>
G	II	Phenylacetic		<i>P. notatum</i>
X	III	<i>p</i> -Hydroxy-phenylacetic		<i>P. notatum</i>
K	IV	Caprylic	$C_7H_{15}CO-$	<i>P. notatum</i>

**Solubility.** Sodium salts of penicillin are soluble in water and alcohol, and insoluble in petroleum ether, ether, and chloroform (19,68). The free acid is soluble in ether, chloroform, amyl acetate, and butanol (3). The sodium salt can be precipitated from a butanol solution by petroleum ether (19). Penicillin X acid is insoluble in chloroform.

**Synthesis.** Penicillin G, identical with the natural penicillin G, was synthesized from *d*-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone in a yield of about 0.1% (159a). The synthesis could not be used as synthetic proof of structure. The same synthesis has been used to form very small amounts of analogs of penicillin differing from the known varieties in the nature of the substituent groups at position 2 of the thiazolidine ring (159a).

**Stability.** Boiling for a few minutes has little effect (probably at a  $pH$  near 6.5); autoclaving at  $115^\circ C.$  for 20 minutes practically destroys it (68). The stability of penicillin is a function of  $pH$  and temperature (17, 52). At  $pH$  2 in aqueous solutions penicillin G is half destroyed in 18.5 minutes at  $24^\circ C.$  and is half destroyed in 255 minutes at  $0^\circ C.$  (16). Hence operations with the free acid are conducted as near  $0^\circ$  as possible to minimize destruction. The destruction seems to be a first-order irreversible reaction (17). At  $pH$  6.0,  $pH$  of maximum stability, penicillin G is half

destroyed in 336 hours at 24°C., and in 103 hours at 37°C. (16). Data on the stability of penicillins at pH 2 is as follows:

Solution of penicillin	Time (min.) at which half destroyed at pH 2, 24°C.
F.....	11
G.....	18.5
X.....	11
K.....	7

Thus G is the most stable, and K is the least stable, form of penicillin. Extrapolation of the measurements (16) to 100°C. indicates that penicillin G at pH 3 is destroyed completely by boiling for 1 minute.

One or more penicillins are formed by the following fungi: *Aspergillus parasiticus* (54), *A. giganteus* Wehn (129), *A. niger* YW (72), *A. nidulans* (72), *A. oryzae* TP (72), *A. flavipes* (72), *A. flavus* (74,110,111), *Penicillium citreo-roseum* (72), *P. notatum-chrysogenum* group (139,140), *P. fluorescens* NCTC 6621 (69), *P. rubens* Biourge NCTC 6643 (69), *P. avellaneum* Thom and Turesson NCTC 3751 (69), *P. baculatum* Westl. NCTC 3956 (69), *P. turbatum* Westl. NCTC 6523 (69), *P. chrysogenum* (152), and *P. notatum* (52,68), growing on a variety of media.

The penicillins are active mainly against certain of the Gram-positive bacteria. They have little action against most of the Gram-negative bacteria, the acid-fast bacteria, and the fungi. The activity of crystalline sodium penicillin G is defined as 1666 units per milligram for all test bacteria (148). Table II gives the activities in units per milligram for the crystalline salts of the other penicillins.

**Toxicity.** Penicillin is relatively nontoxic to mice, the LD<sub>50</sub> being greater than 600 mg. per kg. On the contrary, penicillin is quite toxic to guinea pigs, 4 mg. per day for four days being enough to cause death within six days (138a).

**Pleurotin.** This compound (142a) has the empirical formula, C<sub>20</sub>H<sub>22</sub>O<sub>6</sub>, m.p. 209°C. (dec.), yellow or amber needles, [ $\alpha$ ]<sub>D</sub><sup>25</sup> = -20° in chloroform, absorption peak at 2500 Å; very soluble in chloroform; soluble in alcohol, acetone, ether, and benzene; nearly insoluble in water and petroleum ether; gives no color with ferric chloride, releases iodine from hydrogen iodide. It reacts with potassium cyanide to give a blue color, which is the basis of a chemical method of assay (101a). One mole of pleurotin reacts

with about 1.5 moles of sodium hydroxide to form an acidic substance without antibacterial action. Pleurotin is unstable at pH 6.5 and at pH 3 when heated to 100°C. for ten minutes. Solutions of pleurotin are unstable in light. Pleurotin is active on Gram-positive bacteria and certain fungi. It is formed by *Pleurotus griseus* grown on a corn-steep medium in static cultures. Culture filtrates contain from 100 to 200 mg. of pleurotin per liter of solution. It is not toxic to mice when a single dose is given at 24 mg. per kg. intravenously. Insolubility of pleurotin in saline solution prevented the use of larger amounts.

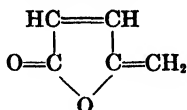
TABLE II  
ACTIVITIES OF CRYSTALLINE SODIUM SALTS OF THE PENICILLINS

Bacterium and strain	Units per milligram*					
	F (148)	F (138a)	Flavi- cidin (74)	K (138a)	X (148)	X (138a)
<i>Staphylococcus aureus</i> FDA 209P	1490				845	
<i>Staphylococcus aureus</i> Heatl.	1440	1465	1400	2300	935	850
<i>Bacillus subtilis</i> , smooth NRRL B 558	970				800	
<i>Bacillus subtilis</i> , rough NRRL B 558	970				1200- 1700	

\* Based on activity of penicillin G of 1666 units per milligram by definition.

**Protactinomycin.** Protactinomycin (76) is a basic substance very soluble in water and soluble in ether. It is precipitated from aqueous solution by picric acid, picrolonic acid, flavianic acid, and other base precipitants. There is little loss of activity after boiling for 10 minutes at pH 2 or pH 7. Protactinomycin is destroyed by boiling at pH 10, but it can be kept at pH 10 for 1.5 hours at 25°C. without appreciable loss. It is produced by *Protactinomycetes* (*Nocardia*) *gardneri* grown on a glucose-agar medium. About 60 mg. crude protactinomycin can be obtained from one liter of medium (76). It is active against Gram-positive bacteria and has little, if any, activity against Gram-negative bacteria, mycobacteria, and fungi (71). At low concentration it lengthens the lag phase of growth of *Staphylococcus aureus* and at higher concentrations it kills the cells (2). It is toxic at 50-100 mg. per kg. for mice intravenously (76). It can be used to cure mice infected with *Streptococcus pyogenes*.

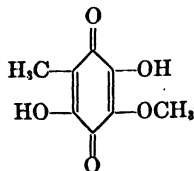
**Protoanemonin.** This substance (13,151) has the formula  $C_6H_4O_2$ :



Protoanemonin, an unsaturated reactive lactone, is very unstable and in concentrated solution rapidly polymerizes to anemonin and higher polymers which are devoid of antibacterial activity. It can be obtained by steam distilling ground leaves and extracting the distillate with chloroform. A 1% aqueous solution was stable for many months at 5°C. Hydrolysis destroys activity. The ring structure is essential for antibacterial activity. Obtained from *Anemone pulsatilla*. Protoanemonin is active against Gram-positive and Gram-negative bacteria, *Mycobacterium tuberculosis*, and pathogenic yeasts. It is too toxic to animals to use in the therapy of infection.

**Puberulic Acid.** This acid (21,123),  $C_8H_6O_6$ , m.p. 316–318°C., is nearly colorless microcrystalline plates, dibasic monocarboxylic acid. The molecule contains two hydroxyl groups and two atoms of oxygen in an unknown combination. The acid reacts with ferric chloride to give a striking brownish red color. It is isolated from the metabolism solution as the nickel complex and is purified through the characteristic colorless crystalline diacetate, m.p. 212°C. It is formed by *Penicillium puberulum* Bainier, by *P. aurantio-virens* Biourge, by the *P. cyclopium-viridicatum* series, and by *P. Johannioli* Zaleski growing on synthetic media. About 100 mg. of acid can be obtained from a liter of culture filtrate. Puberulic acid is active on Gram-positive bacteria only. Toxicity for animals was not reported.

**Puberulonic Acid.** This substance (21,123) has the formula  $C_8H_4O_6$ , m.p. 298°C., bright yellow prisms. Puberulonic acid on neutralization gives a series of color changes suggestive of a pseudo acid. No ester or acetyl derivatives could be obtained. It gives the same ferric chloride color as puberulic acid, forms a nickel complex, and is purified through the sodium salt. It is formed by *Penicillium puberulum* Bainier, by *P. aurantio-virens* Biourge, by *P. cyclopium-viridicatum*, and by *P. Johanniola* Zaleski growing on synthetic media. It can be obtained nearly pure from the nickel precipitate of the metabolism medium of *P. Johanniola*. About 60 mg. of the acid can be obtained from a liter of solution. It has low activity against Gram-positive bacteria. Toxicity for animals was not reported.

**Spinulosin.**  $C_8H_8O_5$ :

3,6-Dihydroxy-4-methoxy-2,5-toluquinone, synthesized (7), m.p.  $201^{\circ}\text{C}$ ., purple bronze plates, optically inactive. Spinulosin is acidic enough to be extracted from acid solution by ether and from the ether by pH 7 buffer. It forms a bluish purple colored solution in alkali and a deep purple colored solution in sulfuric acid (7). It reduces permanganate in the cold (8). Spinulosin is formed by *P. spinulosin* Thom (20) and by *A. fumigatus* Fresenius (8) growing on Raulin-Thom medium. It is active against Gram-positive and Gram-negative bacteria. Toxicity for animals is unknown.

***Spirea aruncus*.** The antibiotic (4) from this plant has the formula  $C_{10}H_{14}O_4$ ,  $[\alpha]_D^{20} = +55.8$  in water, m.p.  $79-80^{\circ}\text{C}$ ., fine colorless prisms, neutral substance, soluble in ether, benzene, and water. It contains one methyl to carbon linkage ( $\text{C}-\text{CH}_3$ ) but methoxyl ( $\text{O}-\text{CH}_3$ ) was absent. It absorbs six hydrogen atoms on catalytic hydrogenation and adds two bromine atoms. It does not reduce Tollen reagent at room temperature, nor does it show ketonic properties. On treatment with cold alkali, the antibacterial properties were destroyed; one acidic group and a ketonic function appeared. The substance is extracted from juice of ground leaves and flowers of *Spirea aruncus*. It is active against Gram-positive and Gram-negative bacteria at the following concentrations:

Bacterium	Minimum concentration, $\mu\text{g./ml.}$
<i>Staphylococcus aureus</i> .....	250
<i>Escherichia coli</i> .....	250
<i>Bacillus proteus</i> .....	250
<i>Pseudomonas pyocyanea</i> .....	500

Toxicity for animals was not reported.

**Streptomycin.** This compound (145) has the hydrochloride,  $\text{C}_{21}\text{H}_{39}\text{N}_7\text{O}_{12} \cdot 3\text{HCl}$ , reineckate dec. at  $162-164^{\circ}\text{C}$ . (cor.) (75), and  $(\text{C}_{21}\text{H}_{39}\text{N}_7\text{O}_{12} \cdot 3\text{HCl})_2 \cdot \text{CaCl}_2$ , dec.  $200-230^{\circ}\text{C}$ . (125),  $[\alpha]_D^{25} = -76^{\circ}$  for 1% aqueous solution of calcium chloride double salt. Streptomycin chloride is soluble in methanol, insoluble in ethanol, butanol, acetic acid, and pyridine. Strept-

tomycin sulfate is slightly soluble in methanol and practically insoluble in ethanol and butanol (38). The Sakaguchi test for guanidine group was positive (38). Streptomycin shows only end absorption below 2300 Å (105). Streptomycin loses half its activity in three hours in 0.1 *N* sodium hydroxide at 25°C. It is somewhat more stable in 0.1 *N* hydrochloric acid being 35% decomposed in six hours at 25°C. Stronger acid or alkali caused more rapid destruction (126). Streptomycin can be hydrogenated to form dihydrostreptomycin which is nearly as active *in vitro* as streptomycin. Activity of the dihydro compound is not inhibited by cysteine; the free or potential carbonyl group is the one hydrogenated (127). The antibacterial action of streptomycin is inhibited by sulfhydryl (58). Carbonyl reagents (*e.g.*, hydroxylamine, thiosemicarbazide) are the most effective inhibitors of activity (60). Streptomycin is relatively stable from pH 1 to 10 but is inactivated by 1 *N* HCl (38). Isolation of streptomycin from culture filtrates depends on its adsorption by activated carbon and elution by acidified alcohol. Streptomycin is produced by a strain of *Actinomyces griseus* grown on peptone-meat extract medium in surface culture and in deep-tank fermenters in which an unspecified medium is used. Streptomycin shows antibacterial activity against a wide variety of Gram-positive, Gram-negative, and acid-fast bacteria (95,145,146,169). It is inactive against obligate anaerobes (23). The activity of streptomycin in terms of "units" is defined as 1000 units per milligram of free base. The reineckate (75) assayed between 800 and 910 units per milligram for free base. The double salt with calcium chloride assayed 750 units per milligram, which is equivalent to 960 units per milligram of free base (125). Streptomycin is not very toxic. A single dose did not kill mice at 500 mg. per kg.

**Streptothricin.** Free base  $C_{13}H_{25}O_7N_5$  (75). The chloride is white with  $[\alpha]_D^{25} = -51.3^\circ$  in water (128). There is only end absorption in the ultraviolet. Streptothricin is a water-soluble organic base that is insoluble in organic solvents. It is adsorbed on activated carbon and is eluted by acidic alcohol (128,160,171). Streptothricin reduces Tollen reagent, cold potassium permanganate, and hot Fehling solution immediately. It is precipitated by picric acid and Reinecke salt. The direineckate decomposes at 192–194°C. (75), the helianthate decomposes at 225–230°C. (128). Streptothricin is stable between pH 1.0 and pH 8.5 (75). Two nitrogen atoms are present as salt-forming basic groups. Streptothricin is free from methoxyl ( $O-CH_3$ ), nitrogen-to-methyl ( $N-CH_3$ ), and hydrolyzable acetyl groups. It contains three or four hydroxyl groups in



addition to two acylatable basic groups. Streptothricin, in contrast to streptomycin, does not give the Sakaguchi test for guanidine groups (75). The antibacterial activity of streptothricin is not affected by sulfhydryl (58). Streptothricin is formed by a strain of *Actinomyces lavendulae* grown on a starch-tryptone medium (160). The culture broth contains from 40 to 80 mg. per liter. Streptothricin is active against Gram-positive and Gram-negative bacteria, mycobacteria, and many fungi. Although the first report (143) on the toxicity of streptothricin indicated that it was relatively nontoxic, later (135) results indicated an  $LD_{50}$  of about 75 mg. per kg. when given intravenously to mice. This value of  $LD_{50}$  was for the delayed toxicity. The immediate  $LD_{50}$  was greater than 250 mg. per kilogram.

**Activity.** The hydrochloride had an activity of 830,000 units per gram when measured with a strain of *Bacillus subtilis* (128). The base had a computed activity of 870,000 units per gram when the reineckate had an activity of 256,000 units per gram.

**Ustin.**  $C_{19}H_{15}O_6Cl_3$  (59), m.p. 184–186°C., ether-soluble acidic substance. Ustin is formed along with at least three neutral compounds (59) by *Aspergillus ustus*. Two of the neutral substances are indistinguishable from ustin by the antibacterial tests used. The activity of ustin increases with increased pH of medium and is decreased by complex media. It inhibits Gram-positive bacteria and mycobacteria but not Gram-negative bacteria (59). Toxicity for animals was not reported. The two substances  $C_{21}H_{17}Cl_3O_6$  (87) (m.p. 185–187°C., square plates, abs. max. 3250 Å) and  $C_{21}H_{18}Cl_2O_6$  (m.p. 214–216°C., fine needles), soluble in ether and chloroform, acidic, no methoxyl group, and at least one more were formed by *A. ustus* grown for 14 days on a Czapek-Dox medium supplemented with 1 g. yeast extract per liter (87). The first compound is an acid, probably dicarboxylic. It was considered (59) to be identical with ustin. The first compound was active against *Mycobacterium ranae* at a concentration of 3  $\mu$ g. per ml. The second compound was one-third as active as the first one (87).

**Viridin.** This antibiotic (27,30) has the formula  $C_{20}H_{16}O_6$ , dec. 217–223°C., colorless, rod-like prisms,  $[\alpha]_D^{19} = -222^\circ$  (1% in chloroform), soluble in chloroform and ethanol, nearly insoluble in ether and camphor. The molecule seems to contain one methoxyl, two active hydrogens, and one methyl group attached to carbon. The addition of phloroglucinol and hydrochloric acid to a very dilute solution of viridin gives a deep reddish violet color which resembles that given by lignin with the reagent. (Gliotoxin

does not give the phloroglucinol color reaction.) Aqueous solutions are relatively stable at pH 3.5. The activity is lost instantaneously at pH 7.5. At pH 6.5 the activity disappears within 24 hours. Gliotoxin is more stable than viridin. Viridin is formed by the strains of *Trichoderma viride* Pers. ex Fries that form a bright yellow pigment when grown on a medium containing nitrate. About 45 mg. of viridin is formed per liter Weindling or Raulin-Thom medium after the trichoderma has grown for four or six days at 25°C. Viridin has little bacteriostatic action. It is very active in preventing germination of the spores of fungi at pH 3.5. A comparison of substances preventing the germination of *Botrytis allii* spores is given below.

Substance	Concentration, μg./ml.
Viridin.....	0.005
Gliotoxin.....	3
HgCl <sub>2</sub> .....	0.5
(C <sub>2</sub> H <sub>5</sub> Hg) <sub>2</sub> HPO <sub>4</sub> .....	0.025

Spores of *Fusarium* spp., *Trichothecium roseum*, and *Cephalosporium* spp., are equally sensitive. Spores of *Penicillium* and *Aspergillus* require 3–6 μg. per ml.

### OTHER ANTIBACTERIAL SUBSTANCES

Several antibacterial substances are poorly characterized. These include the following (usually just one preliminary report has appeared on each):

**Aspergillin.** This (144) is a thermostable substance formed by *Aspergillus fumigatus* NCTC 367. It is active against *Mycobacterium phlei*, but since it is inactive against *Staphylococcus aureus*, it cannot be aspergillic acid or a penicillin.

**Aspergillus** sp. A substance (97) active against *S. aureus* and *Mycobacterium tuberculosis* was formed by an unidentified species of *Aspergillus* grown on a synthetic medium at 37°C. It was said not to be aspergillin, patulin, gliotoxin, or helvolic acid.

**Clitocybine.** Aqueous extract (88) of sporophores of *Clitocybe gigantea* (Fr. ex Sow.) was active against *S. aureus*, *Escherichia coli*, *M. tuberculosis*, *Eberthella typhosa*, *Brucella abortus*, and *Bacillus pyocyaneus* in paper disc tests. Clitocybine could be extracted from the aqueous solution by ether. It was soluble in water, chloroform, ether, acetone, and amyl acetate. Clitocybine seems to be an acidic substance stable in acid and neutral solution and at 40–50°C. but destroyed at 70–80°C. The purified preparation was not toxic for guinea pigs and apparently cured them of tuberculosis.

**Eleocharis tuberosa.** The milky juice (51) of crushed Chinese water chestnuts inhibits *S. aureus*, *E. coli*, and *Aerobacter aerogenes* when used in the cylinder-plate method. It is a fairly thermostable substance insoluble in organic solvents. It may be inactivated by hydrogen sulfide.

**Lichens.** Burkholder *et al.* (34) tested extracts of 42 species of lichens against



Neutral										
Alkali salt	Chemical formula	Mol. wt.	Physical properties	Color	Optical activity	Microscopic appearance	Microscopic appearance	Microscopic appearance	Microscopic appearance	Microscopic appearance
Actinomycin	$C_{42}H_{60}O_{12}$	162	Liquid	+	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	280	115-117 (dec.)	+	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	460	dec. >160	-	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	326	dec. 221	-	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	154	>300	+	Red hydrolyzed	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	342	dec. 209-214	-	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	96	79-80	+	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	198	dec. 217-223	-	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	352		-	-	+	+	+	+	+
Basic										
Actinomycin	$C_{42}H_{60}O_{12}$	250	R <sub>1</sub> †	-	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	581	C	-	-	+	+	+	+	+
Actinomycin	$C_{42}H_{60}O_{12}$	363	C	-	-	+	+	+	+	+

\* Abbreviations and symbols: Y, yellow; C, colorless; M, maroon; R, red; PB, purple-bronze; n, needles; p, prisms; pl, plates; pl<sub>1</sub>, platelets; po, powder; direction of rotation (+ or -); i, inactive.

† Color in aqueous solution unless indicated by minus sign (representing no color).

‡ Reaction occurred (+); no reaction (-).

\*\* Stable in cold and after heating (+); stable only in cold (-).

†† Inactivation (+); no effect (-).

*S. aureus*, *Bacillus subtilis*, and *E. coli*. Twenty-seven gave extracts active against *S. aureus* or *B. subtilis* but none were active against *E. coli*. It was not determined whether the active substance was produced by the alga or by the fungus component of the lichen or whether both were necessary. The relation of these active substances to the lichen acids is unknown.

**Mycetin.** (65). A thermostable substance active against *S. aureus* was extracted by an alcohol-benzene mixture from the agar on which *Actinomyces violaceus* had grown for 15 to 45 days. Activity against other bacteria was not reported.

**Penicidin.** Atkinson, *et al.* (12) have reported on penicidin, which is formed by a unidentified species of *Penicillium* and has been prepared as the crystalline sulfate. It seems to be a basic substance, soluble in ethyl acetate and ether, and slowly inactivated at pH 7.5. It reduces permanganate, is stable at pH 2, and unstable at pH 8. It gives no color reaction with ferric chloride. Penicidin is active against *E. typhosa*. An anti-luminescent method is used as one of the assay procedures (12). Penicidin may be gliotoxin or a closely related substance.

**Penicillium puberulum** Baiffr. A photosensitive compound (36),  $C_{17}H_{12}N_2O_2$ , m.p. 220°C. (dec.), was isolated from the mycelium. The original substance, the diacetyl derivative (m.p. 226°C., dec.), and the dimethyl derivative (m.p. 181°C., dec.) give solutions with blue-violet fluorescence. It has two enolic hydroxyl groups, and the color may be either white or yellow. Absorption bands occur at 2430 and 3740 Å.

**Stilbene.** The compound 4-hydroxy- $\alpha,\beta$ -diethylstilbene (66) was active against Gram-positive bacteria. It inhibited growth of the following bacteria in a streak-plate test at the concentrations indicated in micrograms per milliliter: *Corynebacterium diphtheriae*, 1.6; *Streptococcus pyogenes*, 2.5; *Staphylococcus aureus*, 4; *Diplococcus pneumoniae*, 10. Although this is not a product of a plant, nonetheless it is interesting because of its physiological action in animals.

**Tillandsia usenoides.** An antibacterial substance (176) was extracted from the brown parts of spanish moss with acetone or chloroform. It was active against *S. aureus*, *Pneumococcus* I, II, and III, *S. hemolyticus* C 203, *Hemophilus influenzae* type B, and *Candida albicans*. It was not active against *E. coli*, *Pseudomonas pyocyaneus*, and *H. influenzae* type A.

**Tomatin (lycopersicin).** A substance (92) active against spores of *Fusarium oxysporum* was isolated from the tomato plant.

Several chemical properties of the antibacterial substances have been assembled in Table III. The division into the groups of acidic substances, neutral substances, and basic substances has been maintained. The empirical formulas are those given by one of the authors cited in the references and represent usually results of analysis of the pure substance. A few of the structures, and, hence, the empirical formulas, also, have been proved by synthesis. Several formulas, citrinin, gliotoxin, and patulin, for example, are based upon analytical results and a consideration of degradation products. Probably several of these formulas will be revised after more work has been done with the compounds. The approximate stability of the compounds in acid, neutral, and alkaline solutions are given. Many of

the ferric chloride and hydriodic acid and all the potassium cyanide color reactions were determined by the author. The many blank spaces in the

TABLE IV  
GENERAL BIOLOGICAL PROPERTIES AND METHODS OF STERILIZATION OF ANTIBACTERIAL SUBSTANCES

Substance	Chemical class*	Active against				Toxicity for mice, mg./kg.	Method of sterilization*
		Bacteria			Fungi		
		Gram		Acid-fast			
		+	-				
Actinomycin	b?	+	-		-	0.4	
<i>Allium sativum</i>	n	+	+		+	60	
<i>Arctium minus</i>	n	+	-			90	
<i>Asarum canadense</i> A	n	+	-			5	
<i>Asarum canadense</i> B	a	+	-				
Aspergillie acid	a	+	+	+	+	48	S, B
Biformin	n	+	+	+	+		S, G, B
Cassie acid	a	+	-	+			S, G, B
Chaetomin	n	+	-		-		
Citrinin	a	+	-	+		100	S, B
Dicoumarol	a	+	-				
Fumigatin	a	+	+				D
Gladiolic acid	a	+	-		+		
Glilotoxin	n	+	+	+	+	20	S, G, B
Glutinosin	n?	-	-		+		
Helvolic acid	a	+	-		+	400	S, B
Javanicin	a	+	-	+			
Kojic acid	a	+	+			200	
Litmocidin	a	+	-	+		50	B
Mycophenolic acid	a	+	-		+	500	
Notatin	p	+	+				G
Patulin	n	+	+	+	+	15-40	S, G, B
Penicillie acid	a	+	+	+		300	S, G
Penicillins	a	+	+	+	-	600	S, G
Pleurotin	n	+	-	+		>20	S, G
Protactinomycin	b	+	-	-	-	250	B
Protoanemonin	n	+	+	+	+	Toxic	B
Puberulic acid	a	+	-				S, B
Puberulonic acid	a	+	-				S, B
Spinulosin	a	+	+	+			S, G
Streptomycin	b	+	+	+	-	>500	G, B
Streptothricin	b	+	+	+	+	35	G, B
Ustin	a	+	-	+			
Viridin	n?	-	-		+		

\* Symbols used: a = acid. b = base. n = neutral. p = protein. B = heated to boiling in aqueous or aqueous alcoholic solution and cooled rapidly. D = added to sterile distilled water. G = filtered through sintered-glass filter. S = filtered through Seitz pad.

TABLE V. MINIMUM CONCENTRATION (IN MICROGRAMS PER MILLILITER) OF ANTIBACTERIAL SUBSTANCES INHIBITING GROWTH OF BACTERIA FOR TWENTY-FOUR HOURS\*

[illegible]

TABLE V (continued)\*

Name and strain of bacteria	Neutral								Protein	Basic				
	<i>Allium sativum</i> (41)	<i>Arctium minus</i> (45)	<i>Azadirachta indica</i> (42b)	<i>Chaetomium</i> (167)	<i>Gliotoxin</i>	<i>Patulin</i>	<i>Pleurotin</i> (142a)**	<i>Protaeneumoin</i>		<i>Spiraea aruncus</i> (4)	<i>Actinomycin</i>	<i>Protactinomycin</i> †† (71)	<i>Streptomycin</i> ††	<i>Streptothricin</i> ††
<i>Bacillus anthracis</i>	..	..	0.75	..	0.01	..	5 (78)	..	..	..	0.1 (57)	1	.....	>160 (146)
<i>B. mycoides</i>	..	..	..	..	..	..	..	..	..	..	0.04 (104)	..	4 (145)	4 (145)
<i>B. mycoides</i> † (102)	..	..	..	..	..	0.25	15	3	..	..	..	..	16 (146)	>100 (160)
<i>B. subtilis</i>	8	100	2.5-5	13	0.002	10 (28)	5 (78)	..	..	..	0.024 (104)	..	0.13	50
	..	..	..	..	..	0.5 (141)	5 (141)	..	..	..	..	..	1.6 (141)	2 (141)
	..	..	..	..	..	1.25 (163)	..	..	..	..	..	..	2.7 (146)	3 (146)
	..	..	..	..	..	..	..	..	..	..	..	..	8 (145)	1.3 (160)
<i>B. subtilis</i> † (102)	..	..	..	0.04	..	0.25	4	2	..	..	..	..	0.056 (134)	0.8
<i>Sarcina lutea</i>	..	..	0.75	..	0.001	0.5 (163)	<3 (78)	..	..	..	0.004 (104)	..	10 (145)	10 (160)
<i>Staphylococcus albus</i>	..	250	5	..	..	..	..	..	..	..	0.008 (104)	..	..	..
<i>S. aureus</i>	8	..	..	..	0.002	0.23 (112)	6.2 (49)	..	16 (13)	250 (4)	0.01 (57)	..	66 (145)	5 (160)
	..	..	..	..	..	0.67 (163)	5 (78)	..	..	..	0.008 (104)	..	..	..
	..	..	..	..	..	2.8 (80)	..	..	..	..	..	..	..	..
<i>S. aureus</i> Heatley	..	60	2.5	..	..	0.15	8	0.8	..	..	..	..	..	..
<i>S. aureus</i> Heatley (102)	..	..	..	0.33	..	..	8	..	..	..	..	..	..	..
<i>S. aureus</i> FDA 209	..	250	..	..	..	..	8 (107)	..	..	..	..	..	..	..
<i>S. aureus</i> NCTC 3750	..	..	..	..	..	3 (28)	30 (132)	..	..	..	..	2	..	..
<i>S. aureus</i> NCTC 6571	..	..	..	..	..	..	..	..	..	..	..	2	..	..
<i>Streptococcus pyogenes</i>	12	75	>10	..	..	..	25 (49)	..	..	..	0.001-0.01 (57)	2-7	..	..
<i>S. pyogenes</i> C 203	..	..	..	..	..	..	12 (132)	..	..	..	0.08 (104)	..	..	..
<i>S. viridans</i> NCTC 3166	..	..	..	..	..	0.94 (112)	63 (136)	..	..	..	..	..	0.54 (136)	..
<i>S. viridans</i>	8	1000	>10	..	..	..	30 (132)	..	..	..	..	4	..	..

\* Figures in parentheses are literature references. † Serial dilution in beef extract dextrose. \*\* Activity decreases on further incubation. †† Activity decreases with increase in concentration of bacteria. ‡‡ Preparations varied greatly in degree of purity: (134) based on pure streptomycin base as having 1000 units per mg.; (102) streptothricin, 495 units per mg.



TABLE V (continued)\*

Name and strain of bacteria	Acidic														
	Aspergillus acid	Cassia acid (142c)	Citrinin	Dicoumarol (83)	Fumigatin	Helvolic acid	Kojic acid	Lathocidin (77)	Mycephenolic acid (70)	Penicillic acid	Penicillin G	Penicillin X	Puberulic acid (123)	Puberulonic acid (123)	Spinulosin
<i>Aerobacter aerogenes</i>	30 (96)	..	..	..	..	..	1000 (115)	..	..	..	..	..	..	..	..
	31 (112)	..	..	..	..	..	..	..	..	..	..	..	..	..	..
	20 (177)	..	..	..	..	..	..	..	..	..	..	..	..	..	..
<i>Escherichia coli</i>	30 (96)	..	500 (158)	>40	1000 (163)	2000 (50)	..	1000	>1000	50 (78)	81 (106)	46 (106)	..	..	..
	31 (112)	..	..	..	..	830 (141)	..	..	..	..	..	..	..	..	..
	40 (177)	..	..	..	..	300 (146)	..	..	..	..	..	..	..	..	..
	..	..	..	..	..	800 (163)	2500	..	500	63	16	8	..	250	..
(102)	62	1000	>1000	..	..	..	..	..	..	..	..	..	..	>160	100 (121)
<i>E. coli</i> NCTC 86	..	..	>40 (118)	..	80 (119)	..	..	..	..	10 (118)	..	..	..	..	..
<i>Klebsiella pneumoniae</i>	8 (96)	..	..	..	..	..	1000 (115)	..	..	..	..	..	..	..	..
	8 (112)	..	..	..	..	..	..	..	..	..	..	..	..	..	..
	13	>250	..	..	..	..	620	..	>1000	63	110	250	..	250	..
<i>Mycobacterium phlei</i>	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
	125	16	125	..	..	6 (148)	2500	..	500	63	14	29	..	250	..
	<i>M. phlei</i> (102)	..	..	..	..	..	310	..	250	32	500	500	..	250	500
<i>M. smegmas</i> (102)	16	32	250	..	..	..	..	..	..	..	..	..	..	..	..
<i>Phaenobacterium fischeri</i> † (101)	15 (136)	..	..	..	..	273 (136)	>2500	..	>500	4	>500	>1000	..	128	..
	2	16	16	..	..	..	..	..	..	..	..	..	..	..	..
<i>Pseudomonas aeruginosa</i> (102)	..	>250	250 (158)	>40	..	>300 (146)	300 (93)	..	300 (78)	..	..	..	..	..	..
	250	..	..	..	..	..	5000	..	>1000	1000	500	500	..	500	..
<i>Salmonella enteritidis</i> <i>S. enteritidis</i> Gärtners 125 <i>S. paratyphi</i> <i>S. typhi</i>	24 (112)	..	..	..	..	..	2500 (93)	..	..	..	..	..	..	..	..
	..	..	>40 (118)	..	..	..	..	..	..	30 (118)	..	..	..	..	..
	..	..	..	>40	..	..	..	..	..	..	..	..	..	..	..
	..	..	>100 (118)	..	..	2000 (50)	1200 (93)	..	250	..	..	..	..	..	..

TABLE V (concluded) \*

Name and strain of bacteria	Neutral								Protein				Basic			
	<i>Allium sativum</i> (41)	<i>Asarium minus</i> (45)	<i>Aerum can- dense A</i> (42)	<i>Biformin</i> (142b)	<i>Chaetomin</i> (146)	<i>Gillotoxin</i>	<i>Patulin</i>	<i>Pleurotin**</i> (142a)	<i>Protosmenonin</i> (13)	<i>Spirea aruncus††</i> (4)	Notatin		<i>Actinomycin</i>	<i>Protactinomycin††</i> (71)	<i>Streptomycin††</i>	<i>Streptolysin††</i>
<i>Aerobacter aerogenes</i>	..	.....	.....	..	..	80 (28) 5 (112)	.....	..	..	..	0.04 (104) .....	.....	>100 (164) >200 (170)	>1000 .....	100 (145) .....	30 (160) .....
<i>Escherichia coli</i>	..	>1000 .....	>100 .....	..	>100 .....	>20 (80) 20 (28) 13 (112) 70 (141) 30 (146) 25 .....	12 (49) 3.3 (107) 3 (78) 10 (141) ..... 8 30 (132)	..	30	280 .....	0.05 (104) 1 (57) .....	.....	>200 (164) 200 (170) .....	.....	8 (141) 40 (145) 26 (146) 0.12 (134) ..... 0.03 .....	3 (73) 10 (141) 10 (146) 10 (160) ..... 0.25 .....
<i>E. coli</i> NCTC 86	..	.....	.....	1.7	..	4 (112) 6	.....	>500 .....	..	..	1-10 (57) .....	.....	.....	.....	0.055 (134) 0.13	..... 0.12
<i>Klebsiella pneumoniae</i> (102)	..	.....	.....	1.7	..	1 (146) ..... 4 4	15 (146) ..... 16 1	..	..	..	.....	.....	0.5 (146) .....	.....	10 (145) 3 (146) 0.3 1	6 (146) ..... 7 14
<i>Mycobacterium phlei</i> (102)	..	.....	.....	..	0.02	.....	.....	64 32	..	..	.....	.....	.....	.....	.....	.....
<i>M. smegmatis</i> (102)	..	.....	.....	3.3	..	17 (136) 2	11 (136) 0.5	..	16	..	.....	.....	54 (136) .....	.....	.....	.....
<i>Photobacterium fischeri</i> † (101)	..	>250 .....	.....	0.17	..	.....	.....	..	..	..	.....	.....	.....	.....	100 .....	.....
<i>Pseudomonas aeruginosa</i> (102)	..	.....	53	..	>100	>50 (146) ..... 500	25 (49) 100 (78) 100	..	..	>500 .....	0.024 (104) .....	.....	>50 (146) .....	>1000 .....	1000 (145) 100 (146) 4	>200 (146) ..... 2
<i>Salmonella enteritidis</i> <i>S. enteritidis</i> Gärtners 125 <i>S. paratyphi</i> <i>S. typhi</i>	8 8 8	>1000 >1000 >1000 .....	..... ..... ..... .....	..... ..... ..... .....	..... ..... ..... .....	4 (112) ..... 20 (80) 10 (28)	..... ..... 20 (132) 6 (49)	..... ..... ..... .....	..... ..... ..... .....	..... ..... ..... .....	0.1 (57) 0.01 (57) ..... .....	..... ..... ..... .....	..... ..... ..... .....	..... ..... ..... .....	..... ..... ..... .....	..... ..... ..... .....

† Antiluminescent test; (136) after 0.5 hour at 25°C.; (101) at 24 hours at 18°C.

table show the need for further work. The missing information would be helpful in deciding the identity of an unknown antibacterial substance.

A summary of the general biological properties of the antibacterial substances and methods of sterilization of solutions is given in Table IV. The methods of sterilization are those in the literature and others selected after consideration of the chemical properties of the substances. Usually the acidic and neutral substances can be sterilized by filtration through a sintered-glass filter or through a Seitz pad.

#### IV. Antibacterial Activity

The activity of the antibacterial substances against nineteen species of bacteria has been compiled from the literature and the data are presented in Table V. Work with the purest preparations has been given preference in citation to older, and, sometimes, to the original work. Unless there is a statement to the contrary a "pure" or crystalline preparation was used. Information is scanty about certain antibiotics and is abundant for others, especially the ones that have given promise of usefulness in medicine. To one who must identify antibiotics before they are isolated, this is an unfortunate situation because he needs complete data on each substance.

The data in the table were obtained either by means of a streak-plate (167) or by a serial dilution method (102a,111,112,138). The cup-plate method, although good for determining the concentration of penicillin in a solution relative to the concentration of a standard (67), does not give an absolute value of activity that can be used in characterizing an antibacterial substance. Only a dilution method, such as the streak-plate or the serial dilution, gives usable data. Most of the values reported from the laboratory of Waksman were obtained by the streak-plate method; the other values were obtained by means of the serial dilution method. While both methods have rather large uncertainties resulting from the design of the tests, they probably are adequate.

The media used in the two types of tests are different and may, thereby, modify the action of the antibacterial substances. The influence of alkalinity, salts, and phosphate concentration on the activity of streptothricin (73) can be cited as an example of the effect of composition of medium on activity. Several different media were used in the serial dilution methods. The kinds of media used in testing (when given in the original publications) are as follows: Streak-plate method (104,115,145,146,160,162, 164,166,170). Serial dilution method in (a) beef extract, small inocula (96,102), and large inocula (158); (b) beef heart broth (119,122), small inocula (112,177), and large inocula (50,71,116); (c) beef heart dextrose (123), small inocula (132); (d) nutrient broth (107,158,165); (e) nutrient broth dextrose (57,118,158); (f) 1% tryptone broth, small inocula (134).

Many different strains of bacteria have been used in characterizing antibiotics. If an author did not give the strain of the species of bacterium used, his values are placed on the lines in the table without strain designation. The bacteria in Table V are not the

only ones that have been tested for susceptibility to the antibacterial substances. The results of tests with other bacteria may be found in the papers cited.

The size of the inoculum was usually from  $10^5$  to  $10^6$  bacteria per tube. Small inocula are preferable to large (102a) to eliminate the possibility that bacteria more resistant than the average determined the activity. Although the concentration of the bacteria (2) may affect the serial dilution tests markedly, many investigators do not report the concentration used.

The tests which supplied most of the data for Table V were those using 24-hour incubation at the temperature appropriate for each species of bacterium. The serial dilutions represent bacteriostatic concentrations and not necessarily bactericidal values. With several of the antibiotics, activity decreases upon incubation beyond the 24 hours selected arbitrarily as the incubation time. The *Photobacterium* tests of Rakc, Jones, and McKee (136) were read after 30 minutes, those of the author (101) after 24 hours.

Several of the antibacterial substances and some other plant products have been tested for activity against fungi with the results shown in Table VI.

TABLE VI  
ACTIVITY OF ANTIBIOTIC SUBSTANCES AGAINST FUNGI\*

Fungus	Actinomycin (170)	Chaetomin (141)	Gladiolic acid (26)	Gliotoxin	Glutinosin (31)	Helvolic acid (141)	Patulin	Protoanemonin (13)	Streptomycin (141)	Streptothricin (141)	Viridin (30)
<i>Aspergillus clavatus</i>		> 250		5 (141)		333	300 (141)		> 800	300	3-6
<i>Botrytis allii</i>			2	4 (28)	0.2						0.005
<i>Candida albicans</i>	> 2					100	300 (141)	10	> 800	500	
<i>Dematium</i> spp.						50	70 (141)		800	30	
<i>Fusarium</i> spp.				2-4 (28)		50	40 (141)		> 800	200	0.005
<i>Pythium debaryanum</i>							2 (9)				

\* Values are minimum concentrations of the antibiotic in  $\mu\text{g./ml.}$  which inhibit fungus growth. Figures in parentheses are bibliography numbers.

## V. Identification of Antibacterial Substances

The properties of an antibacterial substance that have been used to characterize it are: its action on bacteria and fungi and on organisms which

may have been made resistant to it, color reactions with various reagents, sulfhydryl inactivation, and special methods such as the inactivation of penicillin by the enzyme penicillinase. The chemical and biological properties of each antibacterial substance, when taken together, form the signature of the substance. It is a signature, to be sure, that resembles others in many details yet when taken as a whole is distinctive. In the discussion in this section the assumption will be made that only one active substance is present in the culture filtrate or concentrate.

One of the most useful properties for classification of antibacterial substances is the ratio of concentrations needed to inhibit *Staphylococcus aureus* and *Escherichia coli*. The relative activity of the substance against these two bacterial species serves to place it in a group that is active primarily against Gram-positive bacteria, in a group active against Gram-negative bacteria also, or in the group that is inactive against bacteria. In this latter group may be found certain substances that are active against fungi but not against the common test bacteria. If the ratio of the minimum inhibitory concentration that is active against *E. coli* to the minimum concentration that inhibits *S. aureus* is less than 16, the substance probably belongs in the group that is active against the Gram-negative bacteria. The problem of identification is considerably simplified when an active substance has been placed in one of these three groups.

Strains of a species of bacteria that have been made resistant or selected for resistance toward one antibacterial substance can provide confirmatory evidence about the identity of substances to which it is not resistant (102a).

Apparently specific chemical reactions are given by only two substances, pleurotin and viridin. Pleurotin (101a) reacts with alkaline cyanide to form a blue color, and viridin gives a reddish violet color with phloroglucinol and hydrochloric acid (30). The ferric chloride test can be applied to culture filtrates to indicate the presence of one or more of the eight antibacterial substances known to give colors with it.

Cysteine inactivation occurs so generally that it would seem to be useful only in distinguishing streptothricin from streptomycin. Reversibility of the sulfhydryl inactivation would indicate whether the antibacterial substance had been permanently changed, as penicillin would be, or whether the inactivation was only reduction to an inactive form, as it may be with gliotoxin.

The temperature and pH range of inactivation is especially interesting because of the ease with which penicillin is inactivated in a slightly acid

solution at room temperature; gliotoxin is inactivated at pH 7. Heating a culture filtrate to boiling between pH 4 and 7 can result in: a decrease in activity, as with penicillin solutions; an increase in activity, as with bi-formin solutions; or no change, as with citrinin solutions.

Penicillin is of such wide occurrence and is of such great importance that a system for identifying it without isolation will be outlined here. In a solution of penicillin the ferric chloride test is negative; the solution has little if any activity against *E. coli*. The activity against susceptible bacteria is completely or nearly completely destroyed by boiling for a minute or two; a solution adjusted to pH 3.0 and kept at 25°C. for two hours loses more than half its activity; at pH 9 and 25°C. the solution loses much less than half the activity in two hours. An apparently specific reagent for the inactivation of penicillin is the enzyme, penicillinase (111). A solution that loses activity on incubation with penicillinase should be suspected of containing penicillins. Penicillin is also inactivated by heavy metals (3). Inactivity toward penicillin-resistant strains of bacteria suggests but does not prove the presence of penicillin. If all the tests listed above indicate the presence of penicillin, then it probably is present. Proof of the presence and the identity of the penicillin must await isolation of the crystalline material.

**Measurement of Components of Mixtures.** The problem of measurement of the individual components of a mixture confronts anyone who works with organisms that form several antibacterial substances. Interest may center in only one component, and its time course of production may be of considerable importance. What, then, are the procedures that have been used to solve the problem?

The preferential destruction (113) of gliotoxin by sodium bicarbonate solution was used to reveal helvolic acid which was then determined by the usual antibacterial method. The activity of the untreated solution, corrected for the helvolic acid present, gave a measure of the gliotoxin. This procedure can be used only when one component is much more stable than the other. It could be applied to mixtures of aspergillic acid and flav-acidin by destroying the flavacidin in acid solution.

Another method is the inactivation of streptomycin in a mixture with streptothricin by cysteine (58). Cysteine inactivation could be applied to other mixtures of cysteine-sensitive and -insensitive substances. Solutions containing penicillins can be treated with penicillinase at pH 7-8 to destroy the penicillins (111). This is a particularly good method to use to establish the presence or absence of penicillins, because, so far as is known, only peni-

cillins are inactivated by penicillinase. Of course, substances like gliotoxin, that are not stable at pH 7.0 at 37°C. would also be destroyed during incubation with the penicillinase.

Chemical methods for the qualitative and quantitative determination of antibacterial substances are not used much because the only specific chemical test suitable for use in mixtures and culture fluids is the cyanide (101a) test for pleurotin. Fairly specific colorimetric (149) and fluorometric (150) methods have been devised for the quantitative determination of penicillin in culture fluids, concentrates, urine, and blood. A chromogenic or a fluorogenic amine is coupled to the penicillin extracted from the aqueous solutions by organic solvents. The methods, measuring total penicillins, do not distinguish between the several kinds; for this reason, the results do not always agree closely with those obtained by bacteriological methods. When a solution of citrinin is treated with hydrogen peroxide and then made alkaline, a deep wine-red color forms immediately (156). The specificity of the reaction is unknown. The Sakaguchi test for the guanidine group proved useful in following the process of concentration of streptomycin (38). Alkaline degradation of streptomycin yields, as one of the products, maltol which gives a brilliant violet color with ferric chloride and which absorbs at 2740 Å. An assay procedure for streptomycin was suggested (147) based upon alkaline hydrolysis and measurement of the amount of maltol by means of the ferric chloride reaction or by the absorption of light at 2740 Å.

A red or brown color obtained when ferric chloride is added to the culture solution indicates the possible presence of one or more of the following substances: citrinin, fumigatin, kojic acid, puberulic acid, and puberulonic acid.

Differential assays by bacteriological methods usually are accurate enough for most purposes, and the development of specific chemical methods has not been necessary. By using several bacteria with greatly differing sensitivities to the components of a mixture, differential assays can be done. If one component is much more active than the other or is present in much greater concentration, it may be possible to dilute the solution so that the less active substance is without effect upon the assay of the more active substance. If only one of the components is active on Gram-negative bacteria, its assay is as simple as assays of solutions containing it alone. A variation of this procedure is to make a strain of bacteria resistant to one component of a mixture and to use it to assay the other component to which it is still sensitive. This could be done with mixtures of strep-

tomycin and streptothricin (168). A strain of *S. aureus* H resistant to streptothricin but sensitive to penicillin, and another strain resistant to penicillin but sensitive to streptothricin can be developed. These two strains can be used to determine penicillin and streptothricin in mixtures. This principle can be extended to other pairs and to more complex mixtures if strains resistant to members of the mixture can be formed. Strains resistant to certain antibacterial substances are formed with great difficulty or not at all, unfortunately. The luminescence of *Photobacterium fischeri* is destroyed by some substances and not by others. It was used to measure small amounts of aspergillic acid in preparations containing large amounts of a penicillin (111) and in assays of solutions containing penicidin (12).

A key for the identification of the known antibacterial substances of natural origin has been constructed, utilizing mainly the data given in Table III (page 486). The substances were divided into 3 groups: those inactive against bacteria, those active against *S. aureus* and not against *E. coli*, and those active against *S. aureus* and *E. coli*. Once this division was made, chemical properties were used; but since other substances, as yet unknown, may key out as a known antibacterial substance, keying does not constitute identification. The compounds with distinctive colors, reactions, and melting points are more easily identified than those without distinctive chemical properties. Some must be in pure form; but, fortunately, these substances are readily crystallized. Confirmatory evidence should be sought in the form of equivalent weights of the acids, optical rotations, absorption spectra, derivatives, reactions for characteristic groups, and relative antibacterial activities. Unfortunately the absolute values of bacterial activities have been measured under so many different conditions and with so many strains of bacteria that they are not as useful as one would like in identifying a substance. The relative activities which are more useful than the absolute activities can be computed from the data contained in Table V (page 490).

### Key for Identifying Antibacterial Substances

- I. Substances active against *Staphylococcus aureus* but inactive against *Escherichia coli*
  - A. Acidic
    1. Brown, red, or violet color with  $\text{FeCl}_3$ 
      - (1) Yellow needles, m.p. 168–171°C.....CITRININ
      - (2) Colorless needles, m.p. 140°C., violet color with  $\text{FeCl}_3$ .....  
MYCOPHENOLIC ACID
      - (3) Red brown color with  $\text{FeCl}_3$ .....  
PUBERULIC ACID OR PUBERULONIC ACID
    2. No reaction with  $\text{FeCl}_3$  or gives a yellow precipitate
      - (a) Unstable in acid solution.....PENICILLIN  
Confirm by following inactivation by acid at pH 3, by cysteine, and  
by penicillinase, by testing against resistant strain of bacteria, and



by determining relative antibacterial activities against several species of bacteria.

(b) Stable in acid solution

(1) Pink to red in alkaline solution.....CASSIC ACID

(2) White crystals, m.p. 204–220°C., optical rotation — .....  
HELVOLIC ACID

(3) Yellow needles, m.p. 230°C., inactivated by cysteine, low activity against *S. aureus*.....ASARUM CANADENSE B

(4) Red in acid and blue in alkaline solution.....LITMOCIDIN

3. Contains chlorine

(1) Colorless crystals, m.p. 184–186°C., contains chlorine..USTIN

B. Neutral

1. Blue color with KCN.....PLEUROTIN

2. No color with KCN

(a) Loses considerable activity after treatment of solution with 5%  $\text{Na}_2\text{CO}_3$  and subsequent neutralization

(1) Colorless crystals without m.p., dec. >160°C., solution acquires greenish fluorescence...ASARUM CANADENSE A

(2) Colorless prisms, m.p. 115°C. (dec.), optical rotation + .....  
ARCTIUM MINUS

(3) Colorless crystals, m.p. 221°C. dec.,  $\text{H}_2\text{S}$  liberated by alkali, active on *E. coli* at from 55 to 230 times concentration that inhibits *S. aureus*.....GLIOTOXIN

(4) Very high activity on *S. aureus*, insoluble in water.....  
CHAETOMIN

(b) Little or no loss of activity on treatment with 5%  $\text{Na}_2\text{CO}_3$  solution

(1) Red color, reduced by hydrosulfite to yellow color,  
m.p. 250°C.,.....ACTINOMYCIN

C. Basic.....PROTACTINOMYCIN

Stable in acid and alkaline solution for a short time, can be boiled at pH 7 and pH 2 for 10 minutes, very soluble in water.

II. Active against *E. coli* as well as against *S. aureus*

A. Acidic

1. No color reaction with  $\text{FeCl}_3$

(1) Melting point 61°C. for acid crystallized from aqueous methanol or water, 86°C. for anhydrous acid, purplish color with dilute  $\text{NH}_4\text{OH}$ .....PENICILLIC ACID

2. Color with  $\text{FeCl}_3$

(1) Colorless prisms, m.p. 93°C., deep red color in alcoholic solution with  $\text{FeCl}_3$ .....ASPERGILLIC ACID

(2) Maroon needles, m.p. 116°C., purple color with  $\text{FeCl}_3$  in alcoholic solution, liberated iodine from  $\text{HI}$ .....FUMIGATIN

(3) Colorless prisms, m.p. 152°C., intense red color with aqueous  $\text{FeCl}_3$ .....KOJIC ACID

(4) Purple-bronze plates, m.p. 201°C., red-brown color with aqueous  $\text{FeCl}_3$ , liberates iodine from  $\text{HI}$ .....SPINULOSIN

## B. Neutral

## 1. Liquid, or with activity destroyed by drying

- (1) Odor of garlic.....ALLIUM SATIVUM  
 (2) Distills with steam.....PROTOANEMONIN  
 (3) Activity destroyed by drying.....BIFORMIN

## 2. Stable solids

- (1) Colorless prisms, m.p. 79–80°C., optical rotation +.....SPIREA ARUNCUS  
 (2) Colorless prisms, m.p. 109–111°C., optically inactive, red color with FeCl<sub>3</sub> after hydrolysis.....PATULIN

## C. Basic

- (1) Inactivated by cysteine, equal activity against *Bacillus mycoides* and *B. subtilis*.....STREPTOMYCIN  
 (2) Not inactivated by cysteine, much more active against *B. subtilis* than against *B. mycoides*.....STREPTOTHRICIN

## D. Protein.....NOTATIN

High activity in glucose-containing culture media, insoluble in organic solvents, stable at pH 3

III. Inactive against *S. aureus*, active against spores of *Botrytis allii*

- (1) Colorless, no m.p., darkens > 300°C., soluble in ether, optical rotation +.....GLUTINOSIN  
 (2) Colorless, m.p. 217–223°C., insoluble in ether, optical rotation –, reddish violet color with phloroglucinol and HCl.....VIRIDIN

## VI. Mode of Action

The mode of action of the antibacterial substances of natural origin is one of the most interesting, most important, and most neglected branches of the entire field of investigation. A serious and persistent study of the antibacterial mechanisms probably would be of more scientific value than the isolation of a half-dozen new active substances. One difficulty, and perhaps the deterrent to such studies, is the state of the present knowledge of the details of bacterial metabolism. The assumption is usually made that the antibacterial substance blocks an essential metabolic reaction with the implication that there is competition between the inhibitory substance and a prosthetic group, or between an essential metabolite and the inhibitory compound for space on an enzyme. Dozens of different enzymes involved in the life processes of bacteria could be blocked. If the antibacterial substance is to be bactericidal, it must block an essential transformation for which there is no alternate pathway. A bacteriostatic substance could be one that blocked the main pathway but left open minor ones which could support life at a reduced rate of growth. There is no *a priori* reason for assuming that only one metabolic step is blocked by an inhibitory

substance, or that the same step is blocked in all sensitive bacteria, or that all concentrations block the same step.

The structure of the bacteria in some way affects the response to an inhibitory material because, while all the antibacterial substances except glutinosin and viridin are active on Gram-positive bacteria, seventeen of them are inactive on Gram-negative bacteria as represented by *Escherichia coli*; and none are active on the Gram-negative group only. Seventeen of the substances have been tested against the acid-fast group of bacteria, as represented by some species of *Mycobacterium*, and sixteen were active. Five of the seventeen substances were active on both *Mycobacterium* and the Gram-positive bacteria but were inactive on the Gram-negative group. This relationship between structure, as indicated by staining reactions, and action of antibacterial compounds needs careful investigation as a part of the general problem of the antibacterial mechanism.

In much of the work with antibacterial substances, an assumption that is implicit but not stated is that the cell membranes of Gram-positive and Gram-negative bacteria are freely permeable to the antibacterial substances and that the site of action is within the cell. If the antibacterial substance is not surface-active, does not remove an essential nutrient from the medium, and does not penetrate the cell membrane of a particular species of bacteria, it should not inhibit growth of that bacteria. The inactivity against certain Gram-negative bacteria of some of the antibacterial substances might be the result of an impermeable membrane barring the way. The bacteria might possess the essential metabolic system that is inhibited by the active substance and yet be insensitive to it because the antibacterial substance was not absorbed by the cell. Insensitive bacteria have not been demonstrated to absorb quantities of the antibacterial substance that would inhibit growth of sensitive strains. The susceptible bacteria differ greatly in their sensitivities to a particular substance. It is just as reasonable to assume that the different sensitivities are a property of the cell membrane as it is to assume that it is a property of the susceptible system within a cell.

The activities of the penicillins increase as their solubilities in lipides increase. Lipide solubility is estimated from the structures of the side chains (characteristic R, Table I, page 478). Penicillin X, that with the lowest lipide solubility, is the least active. The two penicillins, F and flavicidin, that would be expected to have nearly equal lipide solubilities, also have nearly equal activities against *Staphylococcus aureus*. The test of this suggested relation between lipide solubility and activity will be pos-

sible when the structures, chemical properties, and activities of all of the penicillins have been determined.

On the basis of their chemical groups which might be biologically active, the antibacterial substances may be arranged as follows:

**LACTONES:** *Arctium minus*, *Asarum canadense*, citrinin, helvolic acid, patulin, penicillic acid, and protoanemonin.

**$\alpha,\beta$ -UNSATURATED KETONES:** helvolic acid (?), patulin, and penicillic acid.

**$\beta$ -LACTAM (?)**: penicillins.

**HYDROXAMIC ACIDS:** aspergillilic acid.

**QUINONES:** fumigatin, spinulosin, puberulic acid (?), and puberulonic acid (?).

**DITHIO:** gliotoxin.

**SULFOXIDE:** *Allium sativum*.

What are the suggested (39,40,44,48,78) modes of action of these rather diverse reactive groups?

Unsaturated lactones react with sulfhydryl groups (48) to add the RS— to the carbon  $\beta$  to the lactone in  $\Delta^{\alpha,\beta}$  and  $\Delta^{\beta,\gamma}$  lactones. Substitution of a methyl for the hydrogen on the carbon removed the ability to react with cysteine. Citrinin, which may be a lactone, in a concentration of 50  $\mu$ g. per ml. inhibited the lactate dehydrogenase system of *S. aureus* but did not affect that of *E. coli*, a bacterium not sensitive to citrinin (114). The oxygen uptake of *S. aureus* was also decreased by citrinin when lactate or glucose were substrates (114).

Many unsaturated  $\alpha,\beta$  ketones react with sulfhydryl groups to add the RS— group to the  $\beta$ -carbon or to the carbon farthest from the ketone or carbonyl group (48,78). The nature of the substituents on the  $\beta$ -carbon and on the carbonyl-carbon atoms influences the completeness of the reaction with the sulfhydryl groups. The most reactive molecules are those in which an aromatic group is adjacent to the carbonyl group and the  $\beta$ -carbon is unsubstituted. The  $\alpha,\beta$ -unsaturated ketones that reacted most completely with cysteine were also the ones that were most strongly antibacterial (78).

Patulin and penicillic acid are unsaturated lactones and are inactivated by cysteine. They are also unsaturated ketones; whether they react with cysteine as lactones or as ketones is not known. It has been suggested (48,78) that the unsaturated lactones and ketones that have antibacterial activity react with sulfhydryl and possibly with amino groups of essential enzyme proteins. All of the substances listed above under ketone and lactone, with the possible exception of helvolic acid for which there is no data, are inactivated by cysteine.

Gliotoxin which possibly has a dithio structure is reversibly inactivated by cysteine (46). This inactivation may be the result of cysteine's reducing

the gliotoxin. If this is true, the mode of action of gliotoxin could be quite different from that of the unsaturated ketones and lactones which are postulated to combine with sulfhydryl groups. The gliotoxin could oxidize the sulfhydryl groups of the enzymes thereby inactivating them, or it could have an entirely different action. Oxidizing agents, such as hydrogen peroxide and potassium permanganate, inactivated gliotoxin (155). Part of the gliotoxin inactivated by oxyhemoglobin was recovered by acidifying and extracting the gliotoxin with ether (155). A strain of *S. aureus* made resistant to gliotoxin unlike the parent strain grew freely under anaerobic conditions and was avirulent.

Quinones react with sulfhydryl groups and are known to inactivate two sulfhydryl-containing enzymes, urease and succinic dehydrogenase (130), a widely occurring and important dehydrogenase. Cysteine and thioglycolate inhibit the antibacterial action of 2-methyl-1,4-naphthoquinone (53). *p*-Toluquinone (81) slowly combines with glycine in dilute aqueous solution at room temperature. Its *in vivo* action on bacteria, however, is rapid. Other quinones may combine with amino acids or with essential amino groups.

The antibacterial substance from *Allium sativum* (47) is a sulfoxide which reacts with cysteine; and, presumably, it could be listed with the sulfhydryl-inactivating substances.

Compounds that form chelate rings with essential heavy metals should be bacteriostatic and fungistatic. Aspergillic acid is a hydroxamic acid that combines with iron to form a very insoluble red compound (84). It thus removes iron which seems to be essential for the growth of *Mycobacterium tuberculosis*. It may precipitate, or otherwise remove other essential heavy metals from nutrient solutions. The compound, 8-hydroxyquinoline, prevented growth of three fungi (181) by removing the essential element zinc from the culture solution.

Penicillin with its very reactive  $\beta$ -lactam (?) linkage is the most interesting of the antibacterial substances, and yet very little is known about its mode of action. It reacts with cysteine (40) and presumably could be another of the sulfhydryl-reacting substances. Its limited activity indicates that the enzymic systems blocked are essential to a relatively small number of species of bacteria. The feature of the structure common to all of the penicillins seems to be responsible for the antibacterial action with the action modified, in detail, by the character of the side group. This is indicated by the resistance (63) to penicillins F and X of a strain of *S. aureus* made resistant to penicillin G.

## VII. Problems for the Future

About forty antibiotic substances of natural origin have been described in the few years that the intensive search for them has been under way. Of these, five have been synthesized; the structures of six more are known with considerable certainty. The field of antibiotic substances presents problems that are as challenging to the ingenuity and persistence of the organic chemist as any he is likely to find anywhere. The frequency with which totally new structures are found lends zest to what is many times just a routine search for new and useful substances. Some of the antibiotic substances must have relatively simple structures, for the molecules contain too few atoms for them to be complex; yet competent investigators have failed to learn much about their structures. Plants, higher and lower, synthesize dozens of compounds with little structural similarity that seem to have similar actions on other organisms.

The problem for the physiologist is to ascertain the details of the mechanism whereby chemically diverse substances produce apparently identical reactions, to discover the mode of action of the antibiotic substances, and to learn why some organisms are highly sensitive and others almost completely resistant to them. On the answer to these questions, among others, depends rational therapy with antibiotic substances.

The surveys made of the plant kingdom thus far in the search for antibiotic substances have been narrow, as most preliminary studies must be, since everything can not be done at once. If the potentialities of antibiotic substances as specific inhibitors of microorganisms are to be realized fully, compounds active against more than a few common bacteria must be found. This can be done only by using the organism to be inhibited as the test object in the survey. Perhaps the entire vegetable kingdom will have to be searched for inhibitory substances to find those effective against the horde of bacteria pathogenic for plants and animals, of pathogenic fungi, of protozoa, of spirochetes, of bacteriophages, of plant and animal viruses, of yeasts, and even of bacteria causing food spoilage. The procedure will be tedious, if not difficult; but unfortunately, no other yields the information because experience with the few known antibacterial substances indicates that activity against one organism can not be predicted with certainty from the activity against an organism of another class.

Only two of the forty antibiotic substances have much use in medical practice today. The others, however, are not without their scientific value. The determination of their structure not only provides exercise for the skill of the organic chemist but also may afford a clue to the mechanism of inhibition. These compounds also give the chemist models he can modify at will to study details of action. Some of the modified substances may be more active than the parent compounds or inhibitory for a different group of organisms. The physiologist may find among the acid, neutral, and basic antibacterial substances some more useful than the classical iodoacetate, cyanide, and fluoride as inhibitors of enzymes. Other new uses for antibiotics will be found though the field is so new that no one can predict with assurance how it will develop in the future.

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# KIDNEY ENZYMES AND ESSENTIAL HYPERTENSION

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## I. Introduction

A group of related, chronic, degenerative conditions classified as cardiovascular renal diseases lead in today's mortality list. About half a million victims, which is more than one-third the total number of deaths, are claimed each year in the United States. In the age group beyond 45 years, cardiovascular renal diseases are responsible for about four times as

many deaths as cancer and for approximately twenty times as many as either tuberculosis or diabetes.

Cardiovascular renal diseases can manifest themselves in a number of different symptoms and can have a variety of causes. About 50% of all victims in this group, however, suffer from *essential hypertension* the outstanding feature of which is elevation of blood pressure of unknown origin. High blood pressure is often associated with a variety of disease processes, such as disease of the endocrine glands, bilateral renal disease, congenital defects, arteriosclerosis, hyperthyroidism, and aortic insufficiency. In such cases it is often possible to account for the rise in blood pressure as a link in a chain of pathologic events.

In essential hypertension there is an increase in peripheral resistance of the vascular system due to constriction of the peripheral arterioles and, as a compensatory mechanism, the diastolic and systolic blood pressures become elevated. However, the nature of the disturbances that cause this type of vasoconstriction in the absence of recognizable structural disease is not known.

The lack of knowledge as to the origin of this important disease has prevented the development of a rational therapy. There is no cure known for essential hypertension. The best that physicians and surgeons can attempt, at present, is to retard the progress of the disease for some unpredictable length of time, thus possibly prolonging life. But there remains the alarming fact that essential hypertension kills one person about every two minutes in the United States.

Investigation on the pathogenesis of essential hypertension has made promising progress during the last decade. To a large extent this work has been concerned with enzymes and enzyme systems. A discussion of these enzyme studies will form the major portion of this review. Three important findings stand out clearly as nuclei around which most of our present day knowledge of hypertension has been crystallizing. These are: (1) through a relatively simple technic it is possible to produce chronic hypertension in animals (54); (2) renin, a protein obtained from kidneys, elevates blood pressure if injected intravenously (176); (3) renal tissue can transform certain amino acids into pressor amines (84,92,182,183).

## II. Experimental Hypertension

A number of methods have been described for the production of persistent hypertension in animals. Neurogenic hypertension follows the impairment of cerebral blood supply. This can be achieved either through

an increase in intracranial pressure resulting from the injection of a kaolin suspension into the cisterna magna (39) or by ligation of most of the vessels that supply the cerebral circulation (118). Cardiovascular hypertension has been produced by section of the aortic depressor nerve together with bilateral denervation of the carotid sinus (103,104). Endocrine injections, especially of various sterols such as desoxycorticosterone, estradiol, progesterone, and testosterone have been found to produce hypertension in rats (69), and the oral administration of vitamin D to dogs has also been accompanied by elevation in blood pressure (4,75).

Most important, however, has been the perfection of a method to produce hypertension of *renal origin*, because the type obtained resembles to a striking degree the essential hypertension observed in human patients. Paessler and Heinecke (124) found in 1905 that progressive reduction of the amount of renal tissue resulted in elevation of blood pressure associated with cardiac hypertrophy; since that time numerous authors have described a variety of technics for producing hypertension of renal origin. The ingenious method of Goldblatt (54) finally made it possible to obtain with great regularity a marked and persistent elevation of blood pressure in dogs.

In the Goldblatt method hypertension is induced by moderate constriction of both main renal arteries (by adjustable silver clamps) or by the constriction of one main renal artery and extirpation of the contralateral kidney. The Goldblatt procedure is applicable not only to dogs, but also to the monkey, rat, rabbit, goat, and sheep. Historically, it is interesting that Katzenstein (100) in 1905 had already observed a slight temporary elevation of blood pressure after partial occlusion of the renal arteries in dogs.

There are many similarities between human essential hypertension and experimental renal hypertension. To mention a few: In both cases cardiac rate and output, blood volume, and blood viscosity, as well as peripheral blood flow, are normal. Renal blood flow seems to be reduced, while renal excretory function remains normal in the benign phase of both the human and the experimental disease and becomes reduced in the malignant phase. In both types of hypertension cardiac hypertrophy occurs and is chiefly left ventricular.

Most workers in this field agree that human essential hypertension and experimental renal hypertension are closely related, if not identical. Ultimate proof of such a relationship will probably have to await the development of a therapy for experimental hypertension and demonstration of the



control of human essential hypertension by the same therapeutic measures. There is no question that essential hypertension does not originate via constriction of both main renal arteries. The question is rather: Does essential hypertension in human patients originate in the kidney through pathologic changes similar in their end result to those obtained after clamping the renal arteries in dogs?

### III. The Renin-Hypertensin-Hypertensinase System

#### A. RENIN

The name *renin* was suggested by Tigerstedt and Bergman (176) for a substance which they discovered in saline extracts of fresh rabbit kidneys as an agent capable of causing prolonged elevation in blood pressure if injected intravenously. Two or three minutes is required before the maximum rise in blood pressure due to renin is obtained. The pressure remains at its maximum height for several minutes and then declines gradually to normal, usually within fifteen to twenty minutes. Renin is a colorless pseudoglobulin, nondialyzable and insoluble in alcohol and acetone. It is contained in the protein fraction soluble in 0.33 saturated and precipitated by 0.46 saturated ammonium sulfate solution (157). The material shows no loss of activity if its solutions are heated for short periods of time to 56°C., but it is inactivated rapidly at higher temperatures (176). The purest renin preparations so far described were obtained by Katz and Goldblatt (99) and by Schales (157). The various steps used in these methods are outlined in Schemes 1 and 2. Since pure renin has not yet been isolated, yields in purification procedures have to be expressed in activities rather than on a weight basis.

There is no generally accepted "Renin Unit." Schales and Haynes (155) defined one rabbit unit of renin (R.U.) as the amount of renin (expressed as micrograms of nitrogen) that was required per kilogram of body weight to cause a rise in blood pressure of 30 mm. Hg when injected intravenously into adult rabbits. For the assay, at least four unanesthetized animals were used and the blood pressure was measured in the artery of the ear by a membrane manometer (56). The extracts were injected intravenously in the right ear, which had been previously denervated, to prevent any effect on the blood pressure from introduction of the needle.

It is important to inject such amounts of renin as will cause a rise in blood pressure between 20 and 40 mm. Hg as the response in this range is approximately proportional to the amount of renin given. It can be seen from the dose-response curve by Pickering and Prinzmetal (134) that this linearity is not present in the range above 40 mm.

With increasing purity of an extract the amount of nitrogen associated with one R.U. will decrease as less inert proteins are present; the rabbit ear method offers there-

fore a simple way for judging the success of any attempt to purify renin. Schales and Haynes (155) have suggested expressing the *purity* of a given renin preparation as the number of rabbit units per milligram of nitrogen. Independent of its purity the *concentration* of renin in an extract may be expressed as the number of R.U. per milliliter. The purest preparations (157) tested with this method were obtained in a yield of 2500–3600 R.U. per kg. pig kidney and one rabbit unit corresponded to 4–5  $\mu$ g. nitrogen. The highest yields on record (157) are 7500 R.U. per kg. pig kidney with one rabbit unit equal to 34  $\mu$ g. protein nitrogen.

Goldblatt and associates (53) used unanesthetized trained dogs for their bioassays and defined one dog unit (D.U.) as the amount of renin that produced a rise in blood pressure of 30–35 mm. Hg in at least three dogs weighing between 10 and 25 kg. The purest preparation of Katz and Goldblatt (99) was obtained in a yield of 200 D.U. per kg. pig kidney and 1 D.U. corresponded to 7.7  $\mu$ g. nitrogen. Comparative testing of a purified renin preparation in both laboratories indicated that one Goldblatt Unit is approximately equal to five rabbit units as defined by Schales and Haynes.

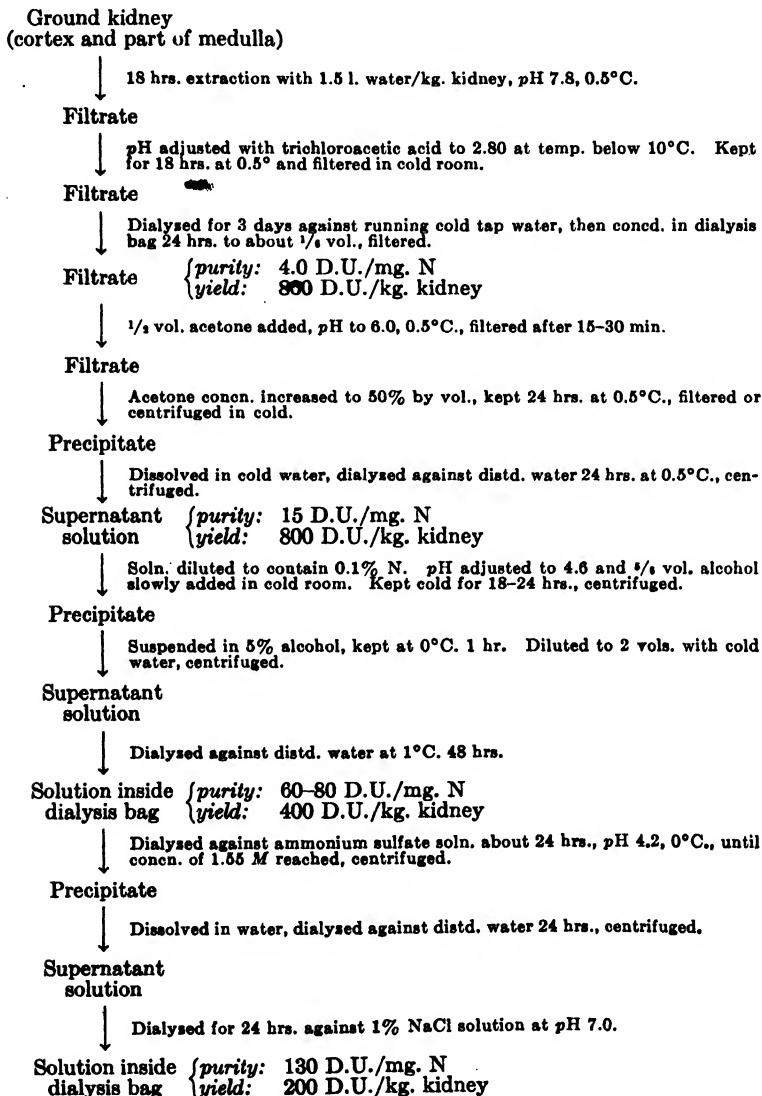
### 1. *Specificity of Renin*

Extracts containing renin have been prepared from the kidneys of a variety of warm-blooded species. For the bioassay of such extracts it is important to be aware of the existence of a specificity, which results in differences of response in the various test animals depending on the source of the renin. Human renin, for example, causes an elevation in blood pressure, whether it is administered to human beings, dogs, or rabbits; but rabbits require much larger quantities per kilogram for a 30-mm. rise than human beings or dogs (157) (see Table I). Pig renin shows equally good activity in dogs and rabbits but is without any effect on the blood pressure of human beings even if large doses up to 1000 R.U. are injected (156,177). Bean (9) reported that chicken renin was active in chickens and ducks, but did not produce a rise in the blood pressure of dogs. Similarly, dog renin, as well as renin from other mammals, failed to alter the blood pressure of chickens or ducks.

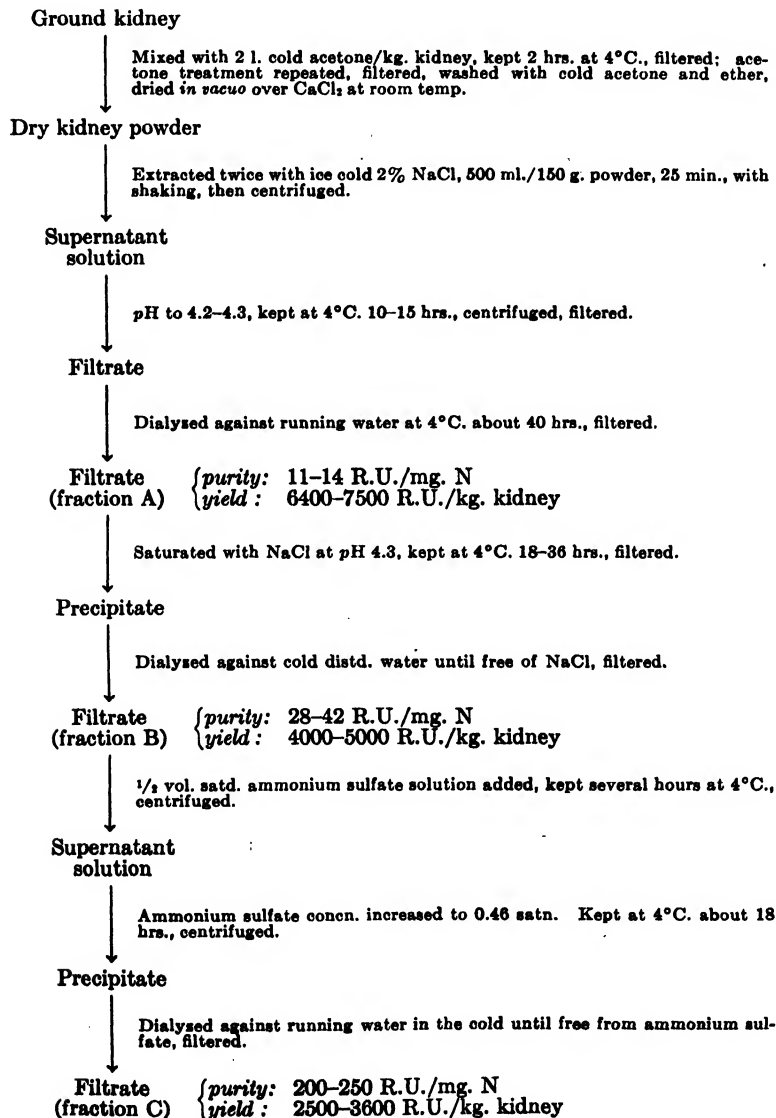
TABLE I  
EFFECT OF HUMAN RENIN ON HUMANS, DOGS, AND RABBITS (157)

Effect on	$\mu$ g. N/unit	Units/kg. kidney	Gram kidney for 1 unit
Humans	42–51*	20000–25000	0.04–0.05
	70–84†	12000–14000	0.07–0.08
Dogs	38–40	25000	0.04
Rabbits	700–775	1300	0.75

\* Ten experiments on three different persons. † Three experiments on one additional person,



### FRACTIONATION SCHEME FOR PREPARATION OF RENIN ACCORDING TO SCHALES (157)



The question of an apparently unsystematic specificity requires further discussion later in connection with the mode of action of renin. It presents rather puzzling problems and has not yet been explained.

## *2. Enzymic Nature of Renin*

Pressor response to renin seems to be a result of its reaction with the peripheral vessels' walls (176). It should, therefore, be possible to produce vasoconstriction in isolated organs, such as the rabbit ear, by perfusion with solutions containing renin. However, it was noticed that experiments (105,107) of this type were successful only when plasma was used as perfusion fluid. Ringer solution containing purified renin did not lead to vasoconstriction in isolated organs.

Kohlstaedt, Helmer, and Page concluded (105):

"These results suggest that renin is an enzyme-like substance which is activated by a kinase-like material contained in the protein fraction of plasma and whole blood." And later they (107) wrote: "These experiments . . . therefore suggest that either whole blood, plasma, or filtrate of laked erythrocytes activates the vasoconstrictor action of renin, or that a latent vasoconstrictor substance in blood is activated by renin."

The enzyme nature of renin was definitely established in Houssay's laboratory by Braun-Menendez, Fasciolo, Leloir, and Muñoz (21,22,116, 117). These authors found that renin is a proteolytic enzyme which liberates "hypertensin" from a serum protein belonging to the pseudoglobulin fraction. The need for plasma together with renin in the perfusion fluid for isolated organs is thus explained. Renin itself has no direct effect on blood pressure but enzymically forms a pressor substance, hypertensin, which in turn causes vasoconstriction. The new conception of the Buenos Aires group found immediate and independent confirmation by Page and Helmer (128), who suggested the name "angiotonin" for the active pressor agent resulting from the reaction of renin with renin substrate.

Renin shows the following properties, characteristic for an enzyme: (1) with fixed amounts of substrate the renin concentration determines the speed of hypertensin formation but not the final yield (22,117); (2) the maximal yield of hypertensin is proportional to the amount of substrate used (22,117); (3) renin shows a typical pH-activity curve (Fig. 1). Optimal activity is found between pH 7.5 and 8.5 (117).

Plentl and Page (136) later investigated the kinetics of hypertensin (angiotonin) formation and showed that under the conditions of their experiments "its rate is first order." These authors overemphasize the importance of this result by stating that it is "a necessary demonstration before

its enzymatic nature can be taken as anything more than a suggestion." And later they (137) added: "Good evidence had suggested that the reaction between renin and renin-substrate is enzymatic, but proof that this is so came from a recent kinetic analysis of the problem demonstrating the reaction to be of the first order." Finally they wrote: "...we have shown that the formation of angiotonin, if enzymatic, should satisfy the requirements of a first order reaction."

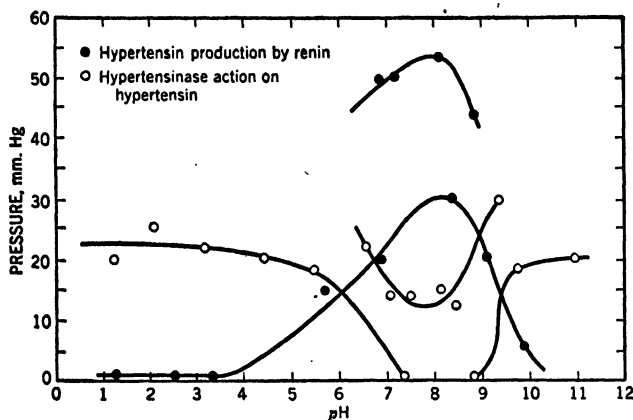


Fig. 1. pH-activity curve of renin and hypertensinase (117).  
Each curve corresponds to one experiment.

The misconceptions expressed in these statements are obvious. It should be remembered that plotting enzymic decomposition of substrate versus time may give a straight line (zero order) or a die-away curve, simulating curves obtained for monomolecular reactions, or anything between these two extremes, or a two-phase curve, representing a combination of both. In no case can the decision whether a process is enzymic or not be based on the shape of time-activity curves.

The question has been raised whether renin is preformed in the kidneys or exists perhaps in an inactive or combined form and is activated or liberated during the extraction procedure. Hessel (83) attempted to show that renin is preformed and not the result of autolytic processes. In his experiments kidneys were frozen in liquid air immediately after removal from the animals. The frozen material was powdered and dehydrated with alcohol or acetone. Treatment with ice cold sodium chloride solutions for short periods of time yielded active extracts. Williams, Groll-

man, and Harrison (190) also froze kidneys in liquid air immediately after removal from the body and dehydrated the material *in vacuo* at  $-30^{\circ}$  to  $-70^{\circ}\text{C}$ . Extracts prepared from the dry powder with sodium chloride solutions had, in a number of experiments, practically no pressor effect. In some instances, however, well-marked pressor responses were obtained. If the dry powders were left in contact with sodium chloride solutions at room temperature for some time, active extracts regularly resulted.

The mechanism of the excretion of renin after the clamping of a renal artery also needs clarification. It has been suggested by Schales (161) that the kidney cathepsins might be involved in this process. If the disturbance of renal hemodynamics results in an oxygen deficit, this in turn would lead to the accumulation of reducing materials, which are known to activate three of the four recognized intracellular kidney cathepsins. A local autolysis by activated proteinases could then open a path for the escape of renin into the circulatory system. J. Bing (14) perfused kidneys with various substances injurious to the capillaries and observed that renin and renal phosphatase were liberated by diffusion through membranes whose permeability had been greatly increased. Bing feels that the liberation of renin from ischemic kidneys is also due to capillary and cell membrane damage.

It should be added here that Shorr and co-workers (191) found recently in renal vein blood of hypertensive dogs (Goldblatt clamp) a vaso-excitor material (VEM). This substance (168b) appeared in the blood within thirty minutes after the application of a clamp to the renal artery. The intravenous injection of 0.5 ml. of heparinized whole blood from such dogs into normal rats resulted in a hyper-reactivity to adrenaline in the terminal arterioles and precapillaries of the mesoappendix. Blood samples from sixty normal dogs had no such effect on the test rats. VEM disappeared from the blood when chronic hypertension had become established. Kidney slices from normal dogs were found to produce VEM *in vitro* only under anaerobic conditions (168c); under aerobic conditions VEM was inactivated. Within 48 hours after the application of a clamp to the renal artery, the kidney elaborated VEM *in vitro* even on aerobic incubation, which is apparently due to the derangement of the renal mechanism for the inactivation of VEM.

Helmer and associates (82a,168a) described the occurrence of a substance of renal origin in the plasma of animals which had a history of hypotension. Helmer's material produced in nephrectomized cats, but not in normal animals, a prolonged rise in blood pressure lasting up to five hours.

A definite statement as to the nature of this substance is premature; this reviewer feels that it might perhaps be identical with VEM.

### 3. Renin Substrate

The name renin substrate was suggested (131,159) for the substance in serum from which renin liberated the pressor agent, hypertensin or angiotonin. Previously, a variety of terms had been used to designate this material, namely, "renin-activator" (106,142), "hypertensinogen" (117), "prehypertensin" (112), "preangiotonin" (112), and "hypertensin precursor" (117). Renin substrate was found to be a pseudoglobulin (117).

TABLE II  
HYPERTENSIN FORMATION WITH VARIOUS FRACTIONS OF BEEF SERUM (159)

A. FRACTIONATION OF BEEF SERUM WITH AMMONIUM SULFATE						
Fraction	1200 ml., protein 7.83%					
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> concn.		pH	Protein pptd., g.	Grams in distd. water	
	Satn.	M			Sol.	Insol.
A.....	0.30	1.25	6.4	21.1	13.6	7.5
B.....	0.41	1.67	6.8	20.4	15.7	4.7
C.....	0.52	2.15	6.7	4.7	3.8	0.9
D.....	0.67	2.76	6.8	27.6	27.6	0

B. PRODUCTION OF HYPERTENSIN WITH BEEF SERUM AND ITS WATER-SOLUBLE FRACTIONS		
Substrate	Units obtained/100 ml. serum at incubation time of	
	12 min.	120 min.
Whole serum.....	260	430
Fraction A.....	170	120
Fraction B.....	450	520
Fraction C.....	40	70
Fraction D.....	30	40

The material from *beef serum* remained in solution (at pH 6.8) in 0.30 saturated (1.25 *M*) ammonium sulfate solution (Table II) and precipitated when the salt concentration was increased to 0.41 saturation (1.67 *M*). These findings by Schales, Holden, and Schales (159) were confirmed by Sapirstein, Reed, and Southard (154).



Plentl, Page, and Davis (142) found renin substrate from *hog serum* to be soluble in 0.45 saturated ammonium sulfate solution (1.85 *M*) and precipitated at 0.51 saturation (2.10 *M*). An electrophoretic analysis of hog serum showed that renin substrate was identical to, or moved with the same electrophoretic mobility as,  $\alpha_2$ -globulin. In a later paper, Plentl and Page (140) suggested the fraction from hog serum precipitated (at pH 6.0) between 0.36 (1.5 *M*) and 0.49 (2.0 *M*) saturation with ammonium sulfate for the production of angiotonin.

The speed of the reaction between renin and its substrate decreases as the temperature of the reaction mixture is lowered (128). Little difference was observed between 25° and 37°C. as incubation temperature, but the incubation time required for maximal yield of hypertensin had to be increased about six- to eightfold when the reaction occurred between 0° and 5°C. (128,154). Strübe and Croxatto (174) reported inhibition of hypertensin formation when methylnaphthoquinone was added to mixtures of renin and its substrate. The concentration of renin substrate in the plasma is markedly reduced after adrenalectomy (23a,49a,94a,112) and is restored by treatment of the animals with desoxycorticosterone.

## B. HYPERTENSIN OR ANGIOTONIN

The pressor substance, hypertensin, resulting from the reaction of renin with renin substrate is apparently a polypeptide. It can be separated from the protein components of the incubation mixture in various ways, for example, by the addition of three volumes of boiling alcohol, which precipitates the proteins while hypertensin stays in solution (22). The pressor principle is soluble in water, glacial acetic acid, liquid phenol, and ethylene glycol, slightly soluble in alcohol, and insoluble in ether, chloroform, and butyl or amyl alcohol (22,117). It is heat stable, dialyzes through cellophane, and can be precipitated with phosphotungstic acid. All investigators agree that hypertensin is fairly stable in acid solution but that rapid inactivation takes place in alkaline media. It was recommended (140), therefore, that the working range with hypertensin be kept between pH 1.5 and pH 8.5. Plentl and Page (140) found that aqueous solutions of hypertensin, if not sterilized by heat or filtration through Seitz filters, were often inactivated within a few hours at room temperature, and they concluded that hypertensin is very susceptible to bacterial action. In the frozen state hypertensin seems to maintain its activity for several weeks, even if it is contaminated. The pressor substance was inactivated by treatment with hydrogen peroxide, bromine water, nitrous acid (128), lead ace-

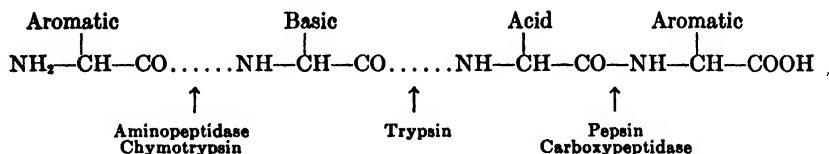
tate, hydroxylamine, and iodine (33). Page and Helmer (128) reported that hypertensin is not precipitated by silver nitrate in acid or alkaline media or by mercuric chloride. Plentl and Page (140), however, precipitated hypertensin with silver nitrate at pH 8 after impurities had been removed by precipitation with silver at pH 1.5; they also reported that hypertensin could be precipitated with mercuric chloride in neutral solution, suggesting that it is associated with the "histidine fraction." Pure hypertensin has not been prepared yet. An earlier statement that a crystalline angiotonin oxalate had been obtained (128) was withdrawn (80) and from subsequent reports (140) it seems reasonable to assume that the claim to have prepared a crystalline angiotonin picrate (128) is no longer maintained.

The purest preparations so far obtained by Page and co-workers are apparently mixtures of polypeptides and amino acids. Adsorption methods have been used for the further purification of such solutions. The pressor substance was adsorbed on Lloyd reagent in acid solution and eluted in alkaline media (128); since hypertensin is not adsorbed by the anionic resin Ionac A and by tricalcium phosphate, these substances were used to remove impurities from hypertensin solutions (32). Edman (41) purified hypertensin by adsorption on aluminum oxide from 90% methyl alcohol and elution with 50–70% methyl alcohol and by precipitation with nitranilic acid (41a). Further purification was accomplished by electrodialysis (42). The isoelectric point was determined as pH 6.8 and the diffusion constant was found as  $1.17$  to  $1.22 \times 10^{-6}$  cm.<sup>2</sup> per sec. Assuming a specific volume of 0.75, this diffusion constant corresponds to a molecular weight of about 2700 (41a,42). Approximately 0.5 to 1.0  $\mu$ g. of the purest preparation produced an appreciable rise in the blood pressure of chloralosed cats (weight 2.5 kg.). On a weight for weight basis the material was 39 times as potent as tyramine phosphate (41a). The best yield on purified product was 30 mg. from 373 liters of horse serum or 2.8% of the activity contained in the crude starting material. Chemical analysis of the purest preparation gave a nitrogen content of 15.5% on an ash-free basis, combustion residue about 4%. The presence of 11 amino acids was shown by paper chromatography: lysine, histidine, glycine, alanine, serine, proline, valine, tyrosine, leucine (or isoleucine), aspartic acid, and glutamic acid. Preliminary data (41a) suggest the following quantitative composition: histidine, 28%; alanine, 3.8%; proline, 5.0%; tyrosine, <2%; aspartic acid, 4.5%; and glutamic acid, 5%.

The presence of peptide linkages in hypertensin was demonstrated

by its inactivation through the action of intestinal aminopeptidase (44), yeast aminopeptidase (29), trypsin, and pepsin (3,22,43,117). Plentl and Page (138), who reinvestigated these reports, subjected hypertensin to the action of four crystalline enzymes, namely, carboxypeptidase, chymotrypsin, trypsin, and pepsin. It was found that these purified crystalline enzymes destroyed hypertensin *in vitro*. An interpretation of these and earlier results on the basis of Bergmann's specificity studies (11) led Plentl and Page to the conclusion that hypertensin contains: (a) a free terminal amino group (required for aminopeptidase), (b) a free terminal carboxyl group (required for carboxypeptidase), (c) one basic amino acid residue which may be terminal, but its carboxyl group must be united in a peptide linkage (required for trypsin), (d) one central dibasic amino acid residue in combination with an aromatic amino acid residue, (e) an aromatic amino acid residue, which may be part of (d) or, if not part of (d), must be terminal with its carboxyl group in peptide linkage (required for chymotrypsin).

The simplest compound satisfying these requirements would be tyrosylarginylglutamylphenylalanine, but it is to be expected that the hypertensin molecule contains additional amino acids at the positions indicated by the dotted lines:



Hypertensin was also inactivated by treatment with tyrosinase (27, 165) which makes it probable that tyrosine is present in the molecule. Plentl and Page (141) synthesized tyrosyllysylglutamyltyrosine and found that this compound (20 mg. per kg.) did not affect the blood pressure of a pithed cat. The synthetic peptide was considerably more resistant than hypertensin toward the action of chymotrypsin and trypsin. Plentl and Page concluded that the sensitivity of hypertensin to tryptic digestion is due to an as yet unknown amino acid arrangement.

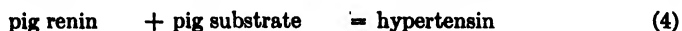
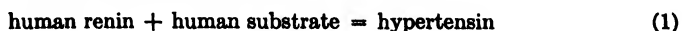
#### *Specificity of Hypertensin Formation in Vitro*

The species specificity of renin was further investigated through studies of hypertensin formation *in vitro* (9,26). Lewis and Goldblatt (112) summarized the results obtained as shown in Table III. There are various

TABLE III  
HYPERTENSIN FORMATION *in Vitro*

Renin from the kidney of		Substrate in the blood of	
Man Baboon Monkey	<p>will react with any of these</p> <p>will react with all yet tested</p>	<p>man baboon monkey</p>	<p>to produce hypertensin, which causes vasoconstriction, or gives a pressor response, in every one of these species yet tested, including the poikilotherms</p>
Cow Horse Pig Sheep Dog Cat Rabbit Rat Dolphin	<p>each will react with any of these, but not with human, baboon, or monkey substrate</p> <p>will react with dog substrate</p>	<p>cow horse pig sheep dog cat rabbit rat dolphin</p>	
Carp Catfish	reaction not yet tested	<p>carp catfish</p>	
Chicken Duck	each reacts with own substrate	<p>chicken duck</p>	
Snake Toad Glomerular fish	no demonstrable homologous or heterologous interactions; therefore no renin or substrate	<p>snake toad glomerular fish</p>	

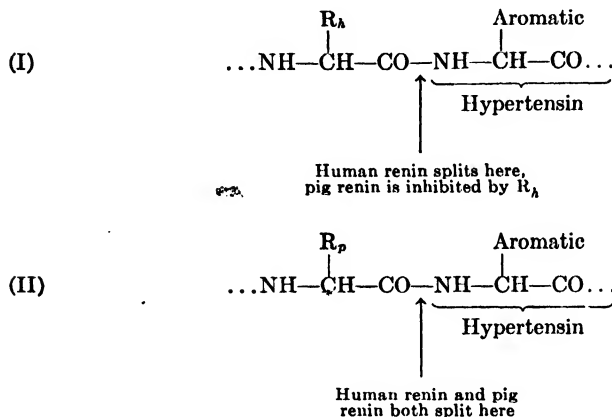
explanations possible for the relations shown in the table. If the discussion is restricted to the following reactions:



it is evident that renin from human kidneys either must have a broader specificity than pig renin or must consist of two enzymes, as it can attack (in addition to pig substrate) the substrate from human blood, which is resistant to pig renin.

If one assumes that the substance, hypertensin, formed in equation (1) is structurally identical with the hypertensin formed in equation (2), one has to conclude that renin substrate of human plasma differs from renin substrate of pig plasma in the amino acid which forms the linkage with

hypertensin. One might illustrate our conception by the following formulas for human substrate (I) and pig substrate (II):



Human renin would thus have the ability to liberate hypertensin, whether or not  $\text{R}_h$  or  $\text{R}_p$  is the characteristic group of the adjoining amino acid. The activity of pig renin, on the other side, would be restricted to those proteins which have hypertensin linked through the amino acid containing  $\text{R}_p$  or other groups, while the presence of  $\text{R}_h$  would prevent hydrolysis.

Equations (2) and (4) show that two enzymes from different species act on the same substrate and form the same reaction product, hypertensin. However, it would be a mistake to draw the conclusion that these two enzymes, pig renin and human renin, are therefore identical. Their non-identity is proved by the equations (1) and (3).

Evidence has accumulated that there are differences in the speed with which renin preparations from various sources hydrolyze the same substrate. Whereas pig renin hydrolyzes dog substrate and rabbit substrate about equally well, as measured by pressor response *in vivo*, human renin seems to react much more slowly with rabbit substrate than with dog substrate. Much larger quantities of human renin had to be given to rabbits to produce the same rise in blood pressure as in dogs (156,157). That these observations are connected with reaction velocities and are not due to an increased destruction of human renin in rabbits was made probable by the finding that the effect of human renin on rabbits persisted much longer (up to one hour) than that of pig renin (157).

## C. PEPSITENSIN

Pepsin hydrolyzes renin substrate with the formation of a pressor substance which was named pepsitensin (28). Furthermore, pressor substances were obtained by peptic digestion of casein, fibrin, serum albumin, and ovalbumin. No pressor substance was liberated when gelatin was treated with pepsin, which probably indicates that aromatic amino acids are essential constituents of pressor peptides.

Although pepsitensin shows many chemical and pharmacological similarities to hypertensin (3), the two substances are not identical. This could be predicted from the fact that pepsin forms pepsitensin but inactivates hypertensin. Both pressor peptides are inactivated by aminopeptidase from yeast (29) and by tyrosinase (27,165). There is some discrepancy in the reports from various laboratories as to the optimal  $pH$  for the formation of pepsitensin. Croxatto and Croxatto (28) stated that renin substrate was subject to peptic digestion between  $pH$  2 and 6 and their graphic accounts showed pepsitensin production at  $pH$  4.5 and 6.0 (28). Helmer and Page (82) found pepsitensin formation when the incubation was carried out at  $pH$  2.0; these authors, however, did not obtain pepsitensin from renin substrate at  $pH$  6.5 in contrast to Croxatto and Croxatto (28). Weber, Major, and Lobb (181) obtained optimal yields between  $pH$  5 and 6. At more acid reactions, only feebly hypertensive or inactive products resulted, or, in some instances, strongly depressor substances were produced. These authors stated also that it was necessary to dissolve the pepsin first in 0.1 *N* hydrochloric acid before it was added to buffered globulin solutions or serum. Experiments to obtain pressor peptides by subjecting globulin solutions to the action of trypsin, papain, cathepsin, and takadiastase were unsuccessful (181).

## D. RENIN AND KIDNEY CATHEPSINS

At least four proteolytic enzymes, known as kidney cathepsins I, II, III, and IV, were found in crude kidney extracts. In their substrate specificity these intracellular proteinases show similarities to pepsin, trypsin, aminopeptidase, and carboxypeptidase, respectively (11). Plentl and Page (139) showed that cathepsins I and IV are not identical with renin. Schales, Holden, and Schales (160) differentiated renin from all four cathepsins. The experimental proof for the nonidentity of renin and kidney cathepsins is summarized in Table IV.

The earlier literature on cathepsins supplies some additional suggestive evidence for the nonidentity of these four proteinases with renin.

TABLE IV  
DIFFERENTIATION OF RENIN FROM KIDNEY CATHEPSINS

Cathepsin	Schales, Holden, and Schales (160)	Plentl and Page (139)
I	Not present in Fraction A (157). Preparation of this fraction is outlined in Scheme 2	Precipitate obtained by treating saline extracts of kidneys with acetone loses about 50% of its cathepsin I activity during 3 weeks at 5°C. while renin activity remains unchanged
II	Fraction C (Scheme 2) contained only 8% of the cathepsin II activity of Fraction A per renin unit	
III	Not present in commercial renin preparation #1000 (S.M.A. Corp.)	Activity greatly decreased on prolonged dialysis (49) without change in renin activity
IV	Not present in Fraction C	

Cathepsins II, III, and IV, for example, require cysteine as activator in order to act on synthetic substrates (49). No need for activators has, however, been observed for the enzymic production of hypertensin *in vitro*. Rapid inactivation of cathepsin I was reported to occur (49) when its solutions were heated to 50°C., whereas renin was resistant to this treatment. However, this marked thermolability of cathepsin I could not be demonstrated by Schales and Holden (163), who found little difference in the rate of hydrolysis of carbobenzoxyglutamyltyrosine before and after heating neutral cathepsin solutions to 50°C. for thirty minutes. Finally, cathepsin II was reported to be unstable even for short periods of time at pH values more acid than 4 (48) whereas renin did not lose activity under these conditions.

#### E. HYPERTENSINASE

Braun-Menendez and co-workers (22) observed that the amount of hypertensin produced when serum globulins were incubated with kidney extracts reached a maximum after about ten to fifteen minutes. When the incubation was continued beyond this period, a gradual disappearance of the pressor principle was noticed (Fig. 2). Page and Helmer (128), who made similar observations, stated that renin destroys hypertensin. The Argentine workers, however, aware of the fact that kidney extracts contain other enzymes beside renin, showed that not renin but a new enzyme.

which they named *hypertensinase*, was responsible for the destruction of hypertensin (22,117).

Hypertensinase occurs in a large variety of tissues. Its distribution in various organs was studied by Fasciolo and co-workers (44) and is sum-

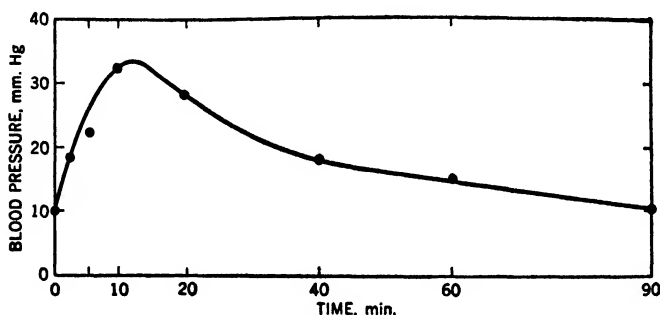


Fig. 2. Blood pressure increases produced by acetone extracts of plasma globulins incubated at 37° C. with kidney extracts for various periods of time (22).

marized in Table V. Optimal activity of hypertensinase was observed between pH 7.5 and 8.5 (Fig. 1, page 521); its action was not suppressed by iodoacetate, sodium bisulfite (44), anaerobiosis, cyanide, octyl alcohol, chloroform, and thymol (117). The enzyme was found to be inactivated

TABLE V

HYPERTENSINASE CONCENTRATION IN VARIOUS TISSUES OF THE DOG (44)

Source	Units* per g. fresh tissue
Kidney	800
Intestinal mucosa	1200-1600
Intestines	400-800
Pancreas	240
Spleen	160-200
Hemolyzed erythrocytes	80-120
Liver	80
Adrenals	40-50
Brain	4-8
Heart	4
Plasma or serum	1
Unhemolyzed erythrocytes	4

\* One unit of hypertensinase was defined as the amount which destroys 0.5 unit of hypertensin in 4 hours at 37°C. and pH 7.4 with a total volume of 10 ml. and an initial amount of 1 unit of hypertensin present (44). One unit of hypertensin was defined as the amount which gives a blood pressure increase of 20-30 mm. Hg in a 10-kg. chloral-injected dog (22,117).



by short exposure (15 minutes) to pH 3.9 at 37°C., conditions which are not harmful to renin and renin substrate.

By the inactivation of hypertensinase in renin substrate and renin solutions before they were mixed, it thus became possible to obtain maximal yields of hypertensin unaffected by prolonged incubation time. An alternative way to prevent the action of hypertensinase in mixtures of renin and renin substrate was described by Sapirstein, Reed, and Southard (154). It was found that the action of hypertensinase is practically arrested if the incubation experiments are carried out at 0°C. while renin continues to form hypertensin, though at a diminished rate.

Little is known about the chemical nature of hypertensinase. The enzyme was not precipitated by dialysis. In fractionation experiments it was found in the fraction precipitated between 30 and 60% saturation with ammonium sulfate. In a comparative study of hypertensinase and proteinase activity of blood plasma (30,31), there was found in various plasma fractions a parallel between hypertensinase activity and hydrolyzing effect on *l*-leucylglycine. These findings were interpreted as supporting evidence for the hypothesis that plasma hypertensinase active at pH 7.3 to 7.8 is an aminopeptidase.

Helmer and co-workers (81), who compared hypertensinase preparations from muscle, liver, and intestinal mucosa with that contained in kidney extracts, found that the greatest activity in kidney preparations was reached at pH 4 while the other extracts showed maximum activity at pH 7. Exposure of kidney extracts to pH 3.5 to 3.9 inactivated only the hypertensinase which had an optimal activity at pH 7 but did not destroy the acid-active hypertensinase (80,81). The existence of an acid-resistant hypertensinase in kidney extracts was also demonstrated by Schales, Stead, and Warren (158), who found 10% of the original hypertensinase activity left (tested at pH 7.4), after incubating extracts for nine hours at 37°C. and pH 3.7. These findings restrict to some extent the statement already made that no loss of hypertensin occurs if kidney extracts and renin substrate are exposed to prolonged incubation after exposure to pH 3.9 for a short period.

The hypertensinase with an optimal activity at pH 4 was destroyed when the acidity was increased to pH 2.0 but under these conditions large losses of renin were observed (137). The ability of hypertensinase from red cells to destroy hypertensin *in vivo* is illustrated in Figure 3. Sapirstein, Reed, and Page (153) hemolyzed about 2% of the circulating erythrocytes in dogs and noted a definite decrease in magnitude and duration of the

pressor response to a subsequent injection of hypertensin. As a 2% hemolysis doubled the hypertensinase content of the plasma and accomplished a 50% reduction of the time required for hypertensin inactivation *in vivo*, the authors concluded that ordinarily only plasma hypertensinase is responsible and available for hypertensin destruction. If the hypertensinases from liver, spleen, kidneys, and other organs were participating normally in hypertensin destruction, doubling of the plasma hypertensinase through hemolysis should have a much less pronounced effect.

Cruz-Coke and collaborators (34) reported that the destruction of hypertensin by kidney extracts was considerably accelerated when oxidized cytochrome c was added. Addition of reduced cytochrome was without effect and the addition of reducing agents such as ascorbic acid or cysteine sharply retarded the inactivation of hypertensin by renal extracts. Cytochrome c did not inactivate hypertensin in the absence of kidney extract. The mechanism of the cytochrome action remains to be explained.

Friedman (47) found less hypertensinase in the renal vein plasma of acutely hypertensive dogs than in the plasma of normal dogs. Later investigators, however, could not demonstrate any difference in the hypertensinase content of normal and hypertensive plasma from human beings (78), dogs (37,153), and rats (153).

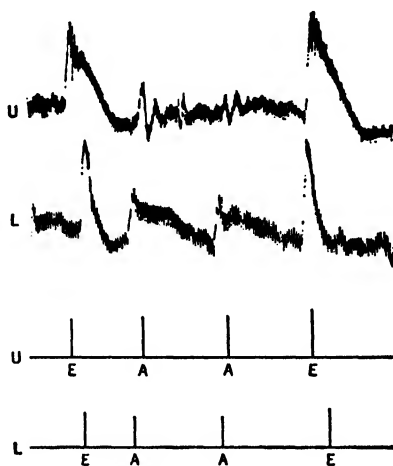


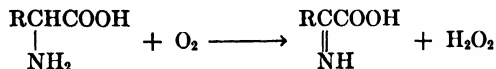
Fig. 3. Effect of hemolysis produced by intravenous injection of distilled water on pressor response to hypertensin (153). Lower tracings (L): responses of blood pressure to injections of hypertensin and epinephrine before intravenous hemolysis is accomplished. Upper tracings (U): same, after intravenous hemolysis has been accomplished. A = 2 ml. hypertensin, E = 0.5 ml. of  $10^{-6}$  epinephrine.

#### IV. The Amino Acid Decarboxylase—Pressor Amine—Amine Oxidase System

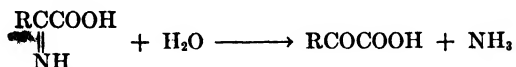
##### A. AMINO ACID BREAKDOWN BY KIDNEY ENZYMES

There exist two different mechanisms for the breakdown of amino acids by kidney tissue. In the presence of oxygen or of a suitable hydrogen

acceptor, *d*-amino acid oxidase (109) and *l*-amino acid oxidase (17,57) dehydrogenate amino acids to imino acids with the formation of hydrogen peroxide:



The imino acids decompose spontaneously in the presence of water to keto acids and ammonia:



Whereas *d*-amino acid oxidase seems to occur in the kidneys of all species investigated, good yields of *l*-amino acid oxidase were obtained only from rat kidneys (17,57); small amounts of this enzyme are, however, present in the kidneys of other animals.

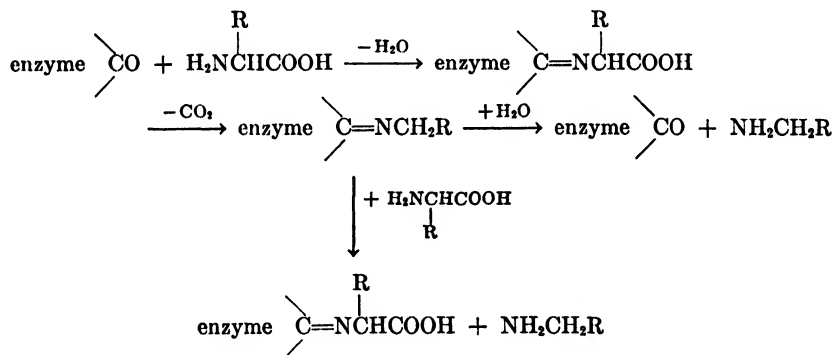
The products of amino acid oxidase activity have no effect on blood pressure. In the alternative pathway for the amino acid breakdown by renal tissue, amino acid decarboxylases convert amino acids to the corresponding amines, some of which are powerful pressor substances.

#### B. FORMATION OF PRESSOR SUBSTANCES BY AMINO ACID DECARBOXYLASES

The ability of various bacteria to decarboxylate a number of amino acids to the corresponding amines was discovered in 1910 by Ackermann and Kutscher (1). Similar enzymes in animal tissues were first described by Werle (182,183) and by Holtz (84,91-93). It was found that these amino acid decarboxylases from kidney, liver, and other organs reacted only with the natural *l* form of amino acids. Marked differences in the amino acid decarboxylase contents in kidneys from various species were noted. The concentration of dihydroxyphenylalanine decarboxylase, for example, was found to be highest in guinea pig kidneys, while pig kidneys were about two-thirds as active and rabbit kidneys showed only one-fifth of the guinea pig kidney activity (92). As extracts from guinea pig and rabbit kidneys were, on the other hand, about equally active toward histidine, Holtz and co-workers (90) concluded that the decarboxylation of dihydroxyphenylalanine and of histidine was caused by two different enzymes. Tyrosine decarboxylase was recognized as a third independent enzyme, which could be adsorbed on kaolin leaving the previously mentioned two decarboxylases in solution (90). The kidneys of some species lack one or

the other of the various decarboxylases. Tyrosine decarboxylase was found in the kidneys of guinea pigs and rabbits but not in the kidneys of rats, pigs, cats, and dogs (85,93,184). Dihydroxyphenylalanine decarboxylase was obtainable from the kidneys of guinea pigs, rabbits, pigs, and dogs, but not from the kidneys of rats and mice (19,92,94). In a later investigation, however, Holtz and Credner (88) found traces of this enzyme in rat kidneys and liver.

The amino acid decarboxylases showed optimal activity at neutral or slightly alkaline reactions and were strongly inhibited by cyanide. Werle (187) concluded from inhibition studies with bisulfite, dimedon, hydroxylamine, phenylhydrazine, and Girard ketone reagent that a free carbonyl group was essential for the activity of these enzymes, or at least for histidine decarboxylase. The decarboxylation probably starts with the formation of an imino acid and proceeds through the following steps (186):



Imino acids are easily decarboxylated (188) and the amine could then be set free either through hydrolysis or through displacement by a second molecule of amino acid. None of the amino acid decarboxylases has been prepared in pure form, but Werle and Heitzer (186) have purified histidine decarboxylase about 35-fold. Recently, pyridoxal phosphate was shown to be the prosthetic group of several amino acid decarboxylases (7,74,162). For optimal yields on amines, incubations with amino acid decarboxylases were carried out under anaerobic conditions (91) in order to prevent the action of amine oxidase, and, in rat kidneys, also that of *l*-amino acid oxidase.

The formation of histamine, tyramine, hydroxytyramine and tryptamine (184) (as a result of tryptophan decarboxylase activity) is easily demonstrated pharmacologically through the action of these amines on blood pressure. Histamine lowers the blood pressure of cats, whereas the other

three amines are pressor substances. Hydroxytyramine is the most effective pressor agent of the three and has an action on the arterial blood pressure of the cat about  $1/35$  of that of adrenaline (8). In addition to the demonstration of their formation by pharmacological testing, tyramine (86) and hydroxytyramine (92) were both isolated from the reaction mixtures as benzoyl derivatives. Amino acid decarboxylase activity was also demonstrated in the living animal. Holtz, Credner, and Koepp (89) found hydroxytyramine in the urine in both free and conjugated form after oral or parenteral administration of dihydroxyphenylalanine to man or experimental animals. Bing and Zucker (16), who injected *l*-dihydroxyphenylalanine into partially or completely ischemic kidneys of cats, observed in most cases a considerable increase in blood pressure when the clamps were removed several hours later and circulation was restored. The injection of dihydroxyphenylalanine into kidneys with normal blood flow (cats) failed to produce any change in the blood pressure. Oster and Sorkin (123) observed, after the intravenous injection of dihydroxyphenylalanine, a marked rise in blood pressure in cats with experimental hypertension but no rise in normal cats or in cats with acute renal ischemia. The same authors reported blood pressure elevations in patients with essential hypertension after intravenous injection of dihydroxyphenylalanine and obtained a much less marked pressor effect in humans with normal blood pressure. A pressor response to the intravenous injection of dihydroxyphenylalanine in normal rats has been reported by Schroeder (166). This was confirmed by E. W. Page and Reed (125), who observed the same response in normal and in hypertensive rats but obtained considerably more prolonged rises in blood pressure when bilateral nephrectomy had been performed. The pressor action of dihydroxyphenylalanine in rats is far from clear if one recalls that only minute amounts of dihydroxyphenylalanine decarboxylase were found in the liver and kidneys of rats (88).

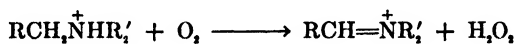
### C. DESTRUCTION OF PRESSOR AMINES BY AMINE OXIDASE

In the presence of oxygen, decarboxylation of amino acids is followed by oxidative deamination to an aldehyde due to the action of amine oxidase. The existence of such an enzyme in animal tissues was first observed in 1928 by Hare-Bernheim (76), who used tyramine as substrate and therefore chose the name tyraminase. It was shown later (20) that a great variety of primary, secondary, and tertiary amines was subject to attack by this enzyme and consequently the name amine oxidase or monoamine oxidase, in contrast to diamine oxidase, was introduced. Only amines with

the amino group at the end of a carbon chain were found to be deaminated. When the amino group was attached to a secondary carbon atom, as in isopropyl amine and *n* ephedrine, deamination did not occur. Such compounds had, however, sufficient affinity for the enzyme to form an addition complex, thus inhibiting the enzyme from further activity (18,20,79).

Monoamine oxidase is inhibited by octyl alcohol, but not by cyanide and, in contrast to diamine oxidase, also not by hydroxylamine. It is irreversibly denatured by urea (13). Optimal activity was found in alkaline solution near pH 8 (185). The enzyme was shown to be strongly activated by pyrrole (12) and this activation was sensitive to cyanide.

The reaction mechanism has been formulated (149) as shown in the following equations:



Substituted amines reacted much slower than the free amines. The reaction velocity for a given amine depends on the partial oxygen pressure and is reduced to about one-third in air as compared to the speed in an oxygen atmosphere (108). Kohn (108) found the enzyme to be associated with insoluble material; Alles and Heegaard (2) also noted activity only in turbid "solutions."

Amine oxidase was found in a variety of tissues (13,20,87,185); the highest concentrations occurred in liver and kidney. The kidneys of rats, however, in contrast to those from man (19), ox, pig, and sheep, seem to contain almost no, or only relatively little, amine oxidase (20,87,92,144, 185).

Since amine oxidase requires oxygen in order to inactivate amines, it was taken into consideration that the restriction of renal circulation in experimental hypertension might make it possible for some pressor amines to escape unoxidized from the kidney and cause general peripheral vasoconstriction. It is not definitely known, however, whether partial constriction of a renal artery to the extent necessary for the production of hypertension creates enough of a deficiency in oxygen to prevent or decrease the action of amine oxidase. The oxygen demand of the kidney is remarkably high. Glaser, László, and Schürmeyer (50) reported that usually 5 to 10%, but occasionally up to 20%, of the total oxygen consumed by the animal organism is used by the kidneys. It seems possible, therefore, that even a small decrease in renal circulation might have a noticeable effect on renal

metabolism. In perfusion experiments with isolated kidneys Bing (15) observed proportionality between the degree of reduction in blood flow and the amounts of pressor amine formed.

Several investigators studied the oxygen consumption of tissue slices from normal and hypertensive kidneys with conflicting results. Gerbi *et al.* (49b) compared the  $Q_{O_2}$  of normal rabbit kidneys and of kidneys rendered "ischemic" by the application of a clamp to the renal artery three hours to six days prior to the respiration experiments, and found the oxygen consumption to be considerably smaller in ischemic renal cortex. Similar results on dogs were reported by Raska (145). Mason, Robinson, and Blalock (113a), however, noticed no difference in the oxygen consumption between kidneys from normal dogs and rabbits and kidneys from animals that had been hypertensive for five weeks to seven months. There was a moderate reduction in the  $Q_{O_2}$  in kidneys from dogs with hypertension of short duration—probably a transient phenomenon following application of the clamp. Oster and Soloway (122a) found no difference in the content of amine oxidase in the kidney cortex from hypertensive and from normotensive human beings in experiments with freshly autopsied material. Raska (145,146) published data indicating that a variety of oxidative enzymes was markedly decreased in hypertensive kidneys. A careful re-examination of this problem is highly desirable. All kidney slices used for respiration studies should be examined for the presence of necrotic tissue (113a), as this would cause erroneously low  $Q_{O_2}$  values.

Contrary to expectations, amine oxidase is apparently not responsible for the inactivation of adrenaline *in vivo*. It was observed (151) that the rate of oxidation of this hormone by amine oxidase is slow, and Richter (150) came to the conclusion that adrenaline is mainly eliminated in the urine as a sulfuric acid ester with an intact amino group. The product of the reaction between hydroxytyramine and amine oxidase is 3,4-dihydroxyphenylacetaldehyde. It is interesting that this aldehyde lowers blood pressure.

## V. Therapeutic Measures against Enzymically Formed Pressor Agents

### A. TREATMENT WITH RENAL EXTRACTS CONTAINING HYPERTENSINASE

If hypertensin is the vasoconstrictor substance in hypertension, it should be possible to lower blood pressure by the administration of hypertensinase. Several investigators (96,129,130) reported favorable results

in the treatment of experimental and essential hypertension with a crude protein fraction of kidney extracts. Helmer *et al.* suggested (81) an assay of the antipressor potency of such preparations *in vitro* by the determination of their hypertensinase contents.

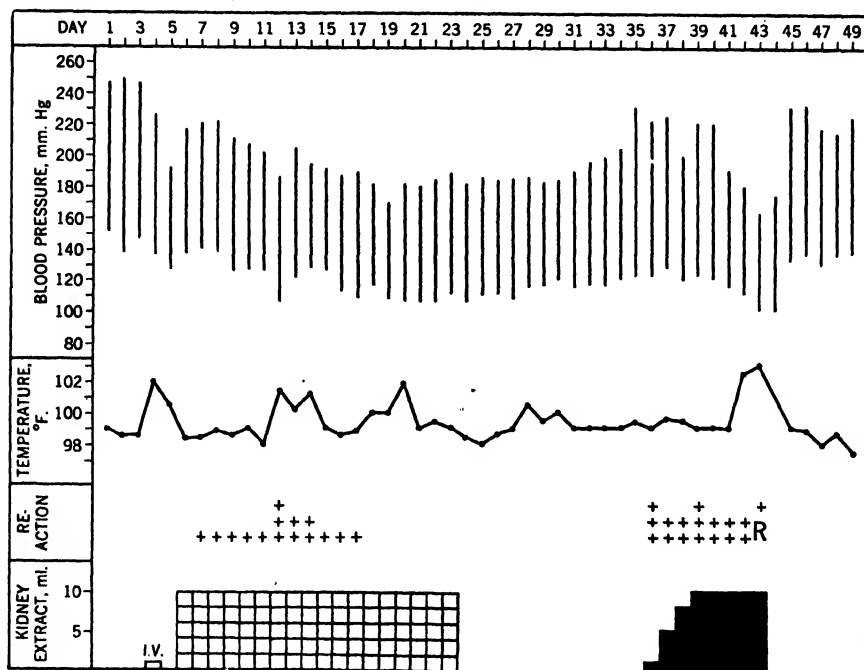


Fig. 4. Effect of intramuscular injection of kidney extract on the blood pressure and body temperature of a 35-year-old patient with malignant hypertension (158). White squares: unmodified renal extract given. Black squares: hypertensinase-poor extract given. The local reactions are graded from 1+ to 4+, depending on their severity. R indicates a shock-like reaction.

Schales, Stead, and Warren (158) confirmed the blood-pressure-lowering effect of certain kidney extracts on hypertensive patients and found considerable hypertensinase activity in their preparations. The daily dose of extract (10 ml.) was obtained from 750 to 1000 g. of hog kidney. It contained an amount of hypertensinase capable of destroying at pH 7.4 in two hours as much hypertensin as is obtainable from 10 to 30 liters of beef serum, using an excess of renin under optimal conditions. These authors observed,



however, that a fall in blood pressure was obtained only after several days of treatment when severe local reactions occurred at the site of the intramuscular injection. The patients showed fever, sweating, weakness, and anorexia. The arterial blood pressure was not lowered in two patients in whom injection of the extract produced neither local reactions nor constitutional symptoms. Intravenous injection of the extract did not lower blood pressure. After destruction of most of the hypertensinase contained in these extracts, the preparation was found to be as effective in lowering the arterial pressure as the original extract had been (see Fig. 4). The authors reached the conclusion that the decrease in arterial pressure was produced by a nonspecific effect of the proteins in renal extracts on the body rather than by specific interference with a renin-hypertensin system. It has been demonstrated before that the induction of fever can lower the arterial pressure in hypertensive patients. Stead and Kunkel (172) reported striking decreases in arterial pressure in two hypertensive patients treated with malaria. Chasis, Goldring, and Smith (23) observed that various pyrogenic agents caused a fall in blood pressure in hypertensive patients, even when the febrile reaction was prevented by the use of pyramidone. Fever as well as a fall in blood pressure seem to be characteristic reactions resulting from the introduction into the body of a variety of foreign materials such as proteins and their breakdown products.

The findings of Schales *et al.* (158) were confirmed by Remington, Cartland, Drill, and Swingle (148) and by Stevens, Kotte, Smith, and McGuire (173). Remington *et al.* fractionated kidney extracts in order to isolate the material which lowered the blood pressure in hypertensive rats. No correlation could be observed between the blood-pressure-lowering activity and the hypertensinase contents of the various fractions.

## II. FORMATION OF ANTIRENIN

Wakerlin and his associates (97,178) reported a fall in blood pressure in hypertensive dogs following daily *intramuscular* injections of crude hog renin preparations. Antirenin to hog renin became demonstrable after several weeks of treatment and reached high titers at the end of three months. The assumption, tempting in the beginning of this work, that the formation of antirenin might be the mechanism through which these extracts affected blood pressure, had to be abandoned as a result of further investigations. There was a poor correlation between the appearance and disappearance times of antirenin and the decrease and return of the blood pressure to hypertensive levels. Furthermore, highly purified renin prepa-

rations produced antirenin, but this was not accompanied by a fall in blood pressure (180). The depressor agent was associated with the nonrenin fraction (177a,178a) and consequently disappeared as the purification of renin progressed.

### C. ORALLY ACTIVE KIDNEY EXTRACTS AND OILS

In order to produce persistent hypertension in animals, it is necessary to restrict the blood flow in both kidneys or to clamp one renal artery and remove the contralateral normal kidney. The presence of a normal kidney seems to have a protective action against the development of severe hypertension. Tigerstedt and Bergman (176) pointed out that the pressor effect of renin was more marked and prolonged in rabbits which had undergone nephrectomy than in normal rabbits. Merrill, Williams, and Harrison (114) found that this increased sensitivity to renin did not appear immediately after removal of the kidneys but developed several days later. The hypothesis was advanced that the body normally contains some substance elaborated by the kidneys which antagonizes the pressor action of renin and which gradually disappears from the body after nephrectomy.

The search for this hypothetical renal antipressor agent led to the preparation of kidney extracts (65,70,71) which lowered the blood pressure in hypertensive rats and dogs (72,77,189) and showed also some activity in several patients with essential hypertension (72). The authors themselves did not regard the results on hypertensive patients as convincing (73). The active fraction was protein-free but could be precipitated by ammonium sulfate. Parenteral administration was frequently accompanied by toxic effects (77) and the material was therefore administered orally. Even then, however, undesirable symptoms were often observed after the administration of large doses (189). There is no proof that this dialyzable, orally active material constitutes an "antipressor factor" of the normal kidney. It remains to be seen whether hypertension is due to a deficiency in an essential humoral agent.

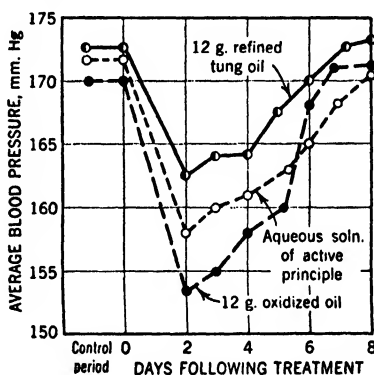


Fig. 5. Effect of tung oil on the blood pressure of hypertensive rats (64). Points are average values for six animals.

The material needed to treat one patient for one week required the processing of about eight hundred pounds of kidneys, so that kidneys cannot be considered a practical source for a therapeutic agent of this type. In a search for better sources, it was found (66) that certain marine oils and tung oil contain a depressor substance (Figs. 5, 6). The active ma-

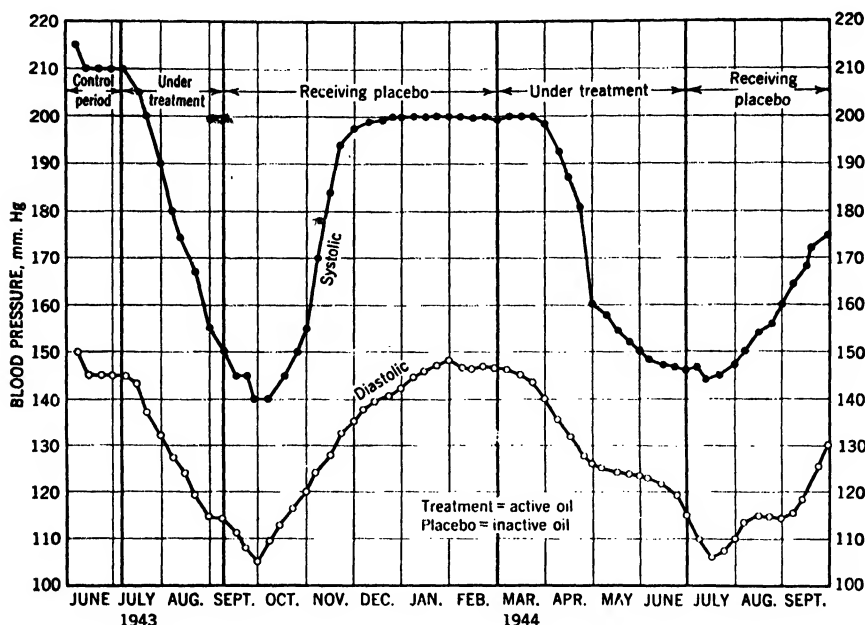


Fig. 6. Effect of oxidized marine oil on blood pressure of a hypertensive patient (64). Each point is the average of at least 42 readings.

terial, apparently an oxidation product of a fatty acid (62), has been obtained in aqueous solution (63,64). In this connection, it is interesting that the depressor effect of large doses of vitamin A preparations (55) was found to be due not to vitamin A, but to constituents of the oil used as solvent for the vitamin (66,115a,179).

#### D. INACTIVATION OF PRESSOR AMINES, MISCELLANEOUS

##### 1. Amine Oxidase

Schroeder (167) treated hypertensive rats and dogs intravenously with a hog liver preparation containing amine oxidase and observed a fall in

blood pressure to normal levels within three to five days. The preparation was somewhat toxic to rats but not to dogs. Since many impurities were present, the author hesitated to conclude that the depressor action was due to the activity of amine oxidase. A preparation in which the enzyme had been inactivated did not affect blood pressure.

Oster and Soloway (122a) injected suspensions of amine oxidase from hog kidney intramuscularly into hypertensive rats and found no significant lowering of blood pressure. This result might be due to the inability of the enzyme to enter the circulatory system from the site of administration. Philpot (132a) reported that *o*-cresolindophenol can replace oxygen as hydrogen acceptor in the reaction between amines and amine oxidase. Oster and Soloway (122a) therefore treated hypertensive rats intramuscularly with 10 mg. *o*-cresolindophenol in aqueous solution, reasoning that it might be the lack of oxygen and not the absence of amine oxidase in the ischemic kidney that prevented the oxidation of pressor amines. They observed a decrease of 30–40 mm. Hg in the blood pressure of three out of six rats, whereas the blood pressure of six normotensive control rats remained unchanged after the injection of this substance.

## 2. Tyrosinase

Mushroom extracts containing tyrosinase were administered intravenously to hypertensive rats and intravenously or intramuscularly to hypertensive dogs (165). Lowering of blood pressure occurred in hypertensive animals, whereas the pressure in normotensive animals showed no significant change. Subcutaneous injection of mushroom extracts decreased the blood pressure of hypertensive patients (164) and this effect was considered to be due to the destruction of hypertensin or of phenolic pressor amines by tyrosinase.

Chasis, Goldring, and Smith (23) found that the treatment of hypertensive patients with tyrosinase preparations resulted in local reactions and constitutional symptoms, such as persistent fever, malaise, prostration, anorexia, dehydration, and loss of weight. The blood pressure fell only when fever and local reactions occurred but it tended to return to the control level as the local reactions subsided and the body temperature returned to normal. Final proof that the depressor effect of mushroom extracts was not due to a specific action of tyrosinase came from observations of Prinzmetal *et al.* (143), who obtained significant depressor effects with heat-inactivated tyrosinase preparations.

### 3. Treatment with Quinones

Soloway and Oster (171) demonstrated *in vitro* inactivation of pressor amines by quinones and quinone precursors. Various quinones also showed a marked depressor effect in hypertensive rats (46,122) after subcutaneous and oral administration. Schwarz and Ziegler (168), comparing the activity of eighteen paraquinones in rats, found that only those having one hydrogen and one alkyl group in ortho position were effective in reducing blood pressure. The corresponding hydroquinones were inactive.

Experiments with hypertensive dogs showed that the quinones were extremely toxic and their use sometimes caused the death of the treated dog (121). It is probably for this reason that no studies of the effect of quinones on human patients have been made. A search for less toxic, related compounds revealed that 1,4-cyclohexanedione had a moderate and delayed depressor effect and was apparently well tolerated (120).

### 4. Cytochrome c

The observation that oxidized cytochrome c accelerated the destruction of hypertensin *in vitro* (34) was followed by a study of the effect of cytochrome c on hypertensive rats (35). Intravenous injections of oxidized cytochrome c were given to sixteen hypertensive rats. The blood pressure fell to normal in eight rats, was appreciably lowered in five, and was not affected in three animals. Oxidized cytochrome c did not decrease the blood pressure in normal rats. Reduced cytochrome c was ineffective in hypertensive animals. Proger (143a) gave 50 to 100 mg. cytochrome c intravenously each day to a few patients with moderate to severe hypertension. There seemed to be no significant effect.

### 5. Low Protein Diet, Restricted Salt Intake

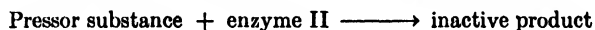
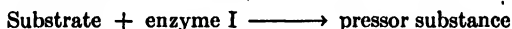
Kempner (101,102) studied the influence of a rice diet on kidney disease and hypertension. The diet used contained in 2000 cal. about 5 g. fat, 20 g. protein derived from rice and fruit, and not more than 0.15 g. sodium. Of 222 patients with hypertensive vascular disease, 62% improved. The mechanism by which these dietary measures lower blood pressure is not fully understood. Grollman and Harrison (67) consider the restriction of the sodium intake as the decisive factor. They found that the feeding of potatoes, peanuts, soybeans, or rice led to a decrease in the blood pressure of hypertensive rats. No decline in pressure was observed, however, if 1-2% sodium chloride was added to the diet. Potassium chloride did not interfere with the lowering of the blood pressure. In six

human hypertensive patients, reduction of the sodium intake lowered the blood pressure to normal levels in two, reduced it moderately in three, while one patient remained hypertensive (68).

Kempner reasons that a low protein diet reduces the amount of work required from the kidney (amino acid metabolism) and thereby decreases the oxygen demand. If insufficient oxygen supply is the cause for production and release of renal pressor agents, decreasing the oxygen demand should leave relatively more oxygen available to counteract the production of pressor substances. There is some experimental evidence that the reverse procedure, namely, feeding a diet rich in proteins or protein breakdown products, can cause an increase in blood pressure. Martin (113) by giving 5-10% *l*-tyrosine as a dietary component produced in rats a syndrome characterized by hypertension.

## VI. Do Kidney Enzymes Cause Hypertension?

In the preceding sections facts were presented which show that the mammalian kidney contains two independent enzyme systems, both of which are capable of forming and destroying pressor substances. These processes can be summarized by two simple equations:



In the light of the available evidence one might be tempted to accept the theory of the renal origin of hypertension and to visualize perhaps a disturbance in the balance between these two enzymic processes as the driving force behind a vicious circle of pathological events. However, the following discussion will show that the problem of the pathogenesis of hypertension has not yet been solved.

### A. EXPERIMENTAL HYPERTENSION

Hypertension can be produced by partial constriction of both renal arteries, by constriction of one renal artery and extirpation of the contralateral kidney, and by a variety of other procedures (127), which all seem to result in a reduction of the expansile pulsation of the kidney. The fact that hypertension can also be produced by transplanted kidneys (24,51), that it may be produced or persist after denervation of the kidneys or after extensive sympathectomy (40), indicates that the nervous system plays no part in establishing this hypertension. However, no rise in blood pressure is observed if the renal vein is clamped simultaneously with the con-

striction of the renal artery (52). This observation strongly suggests a humoral mechanism in the production of experimental hypertension in which a substance or substances from the kidney must be allowed to enter the circulatory system.

The presence of renin in the systemic blood of Goldblatt dogs was actually demonstrated by Dell'Oro and Braun-Menendez (36), but only during the initial period in which the blood pressure was rising abruptly. Once the pressure had reached its maximum and chronic hypertension had become established, it was no longer possible to find renin in the systemic blood. Pickering, Prinzmetal, and Kelsall (135) recovered renin from rabbit blood ten to fifteen minutes after its intravenous injection, but they found no renin in the blood of animals with hypertension of two to five months duration. Such results do not support the view that it is the *continuous* excretion of renin from the ischemic kidney, followed by a steady production of hypertensin, which maintains chronic peripheral vasoconstriction in these animals.

Collins and Hoffbauer (25) transfused blood from large hypertensive dogs into small normal dogs in amounts up to 20% of the body weight of the recipients. Even when the blood was coming from the renal vein of the donors and when the kidneys of the recipients were tied off, these authors did not observe an elevation of blood pressure in the normal dogs. Katz and co-workers (98), who repeated the transfusion of blood from renal hypertensive dogs, used nephrectomized animals as recipients in order to exclude a lack of response due to the presence of normal kidneys. A total of 785-2400 ml. of blood was exchanged in 200- to 300-ml. portions in each experiment and blood pressure determinations were made one hour after each cross transfusion. No rise in blood pressure was noted in any of the nephrectomized dogs. Solandt *et al.* (170) used a special cross-circulation technic which made it possible to exchange approximately three liters of blood per hour in each direction between renal hypertensive and nephrectomized or normal animals. These authors observed that the blood pressure of the nephrectomized animals was invariably raised, whereas that of normal animals remained either unchanged or was lowered. A search for hypertensin (110) in the plasma of animals at the height of the pressure rise due to the administration of renin gave negative results. Landis *et al.* concluded that hypertensin apparently disappears so rapidly from the systemic blood while producing vasoconstriction that it cannot be detected in the perfused rabbits ear preparation.

Further evidence against accepting renin as the pressor substance

responsible for the hypertension in experimental animals was presented by Taggart and Drury (175). Their work was based on an early observation (176) that in all animals a tolerance to renin is rapidly developed if repeated injections are given at short intervals. This phenomenon, which has been given the name tachyphylaxis, is mainly but not exclusively (126) due to the exhaustion of renin substrate in the circulating blood so that no further hypertensin formation followed by a rise in blood pressure can occur. Taggart and Drury found that rabbits with experimental hypertension showed tolerance to renin after twelve to fourteen injections, as did a control series of normal rabbits. On further injections of renin, the blood pressure of the hypertensive animals did not rise but remained at the preinjection, hypertensive level. It is difficult to find a satisfactory explanation for the lack of response to the intravenous administration of renin in these animals without revision of the concept that the maintenance of their hypertension is the result of a continuous response to renin coming from their own kidneys.

Experiments by Ogden *et al.* (119) have contributed material which might permit a reconciliation between the apparently contradictory facts enumerated above. Particularly, the following observations are of importance for our problem:

(1) Patton, Page, and Ogden (132) produced hypertension in rats through careful adjustment of the degree of occlusion of one renal artery. In animals with hypertension of short duration, removal of the affected kidney was followed by immediate return of the blood pressure to normal. If nephrectomy was performed after hypertension had been established for two months, only a moderate lowering in blood pressure occurred. Rats with hypertension of long standing remained hypertensive despite the removal of the affected kidney. Similar experiments by Pickering (133) with rabbits and by Grollman (64a) with dogs confirmed the results obtained by Patton *et al.*

(2) Reed *et al.* (147) treated hypertensive rats with enough Nembutal to produce narcosis and anesthesia. The blood pressure of rats with hypertension of long standing dropped to normal levels, but Nembutal did not lower the blood pressure of newly hypertensive rats. Nembutal affects chiefly the nervous system. Yohimbine, which is known to reverse the action of adrenaline and to block the action of the sympathetic nervous system, also lowered the blood pressure of rats with hypertension of long standing (147) and was active in hypertensive dogs (95), but not in newly hypertensive rats.



(3) Sapirstein and Reed (152) studied the effect of two dioxane derivatives, Fourneau 883 and Fourneau 933, on hypertensive rats. Like yohimbine, F 883 (diethylaminomethylbenzodioxane) is both sympatholytic and adrenolytic while F 933 (piperidinomethylbenzodioxane) is only adrenolytic (5,6). The sympatholytic-adrenolytic drug, F 883, lowered the blood pressure of sixteen late hypertensive rats to a nearly normal level but did not change the blood pressure of normotensive rats. The adrenolytic compound, F 933, had no effect on the blood pressure of late hypertensive rats.

What conclusions can be drawn from the results of these experiments? Evidently, it is an established fact that there seems to occur with the passage of time a change of the mechanism which keeps the blood pressure elevated in these animals. Experimental hypertension has apparently two phases. In the beginning of the disease, a chemical agent coming from the kidney produces peripheral vasoconstriction. After a while and for reasons unknown, the disease enters a second phase. The renal mechanism subsides and sympathetically mediated hypertension becomes established. Lack of response to F 933 excludes hyperfunction of the adrenal medulla in this second phase, which is properly classified as "neurohypertension of renal origin" (119). Success or failure to find renal pressor agents in the systemic blood of hypertensive animals depends therefore on the timing of the experiment. Failure to find renin or hypertensin in the plasma when the second phase of the disease has been entered, does not disprove the importance of the kidneys in the early phase of the disease.

#### B. ESSENTIAL HUMAN HYPERTENSION

The circulatory status of patients with essential hypertension resembles fundamentally the hypertension produced in animals. There is also clinical evidence that the kidneys are diseased in many patients with hypertension. Fishberg (45) and Bell and Clawson (10), as well as Moritz and Oldt (115), have found organic arteriolar disease in the kidneys of a majority of patients who had essential hypertension with or without signs of disturbed renal excretory function. Furthermore, renal ischemia was found to be present in many hypertensive patients (169). This ischemia seemed to be the result of the presence of vasoconstrictor substances in the blood, since it was readily reversible by agents which produce renal hyperemia in normal persons. Smith, Goldring, and Chasis (169) investigated the impairment of renal blood flow in 21 hypertensive patients. In none of these patients did the authors find *unilateral* impairment of the renal blood flow-

Smith and co-workers interpreted this result as evidence against the theory that essential hypertension starts with an obstruction to renal circulation. It is their opinion that such an obstruction would not be distributed symmetrically and would not affect the renal blood flow symmetrically. The final conclusion of these authors is:

"It seems to us therefore...that the theory of primary renal origin is unproved. So far as the genesis of essential hypertension is concerned, the kidney appears to be the victim rather than the culprit."

This reviewer feels that there is no serious discrepancy between the opinions expressed by Smith and his school and those of other investigators in this field. Experimental hypertension does not begin with the excretion of renin or a renal pressor amine into the circulating blood. It starts with a constriction of the renal arteries by a clamp provided by the investigator. Similarly, one could assume the production of an agent anywhere in the human body, perhaps even in the kidney, which acts as "chemical clamp." This agent would initiate changes in the kidneys, comparable to the symmetrical application of numerous Goldblatt clamps to the renal arterioles. From then on the course of essential hypertension would parallel that of experimental hypertension.

The search for a pressor substance in the blood of hypertensive patients has yielded many conflicting reports, too numerous to be discussed here. It seems to be quite difficult to avoid the appearance of pressor substances even in blood taken from normotensive persons (111). Dexter and Haynes (38) confirmed in hypertensive patients the earlier results of Dell'Oro and Braun-Menendez (36) on hypertensive dogs. As in dog blood, renin was found in human blood only when the blood pressure was rising acutely; none was found in the blood of thirteen patients with chronic essential hypertension of all degrees of severity. Taquini and Fasciolo (175a) also found no renin in the plasma of twenty-three patients with essential hypertension. Hypertensin was recovered from the plasma of patients to whom the pressor agent had been given intravenously (58); it was not found, however, in ultra filtrates of plasma from hypertensive patients (58).

Gregory and co-workers (58-61) have presented further evidence to show that the renin-hypertensin system is apparently not responsible for the maintenance of *chronic* essential hypertension in man. A profound drop in blood pressure was observed in patients with essential hypertension during spinal anesthesia. During this period, however, there was no change in the response of these patients toward the injection of hyperten-

sin. As in the case of chronic experimental hypertension, one is again inclined to conclude that not hypertensin but a mechanism of central nervous system origin maintains elevated blood pressure in these patients.

### C. CONCLUSIONS

The experimental facts accumulated during the last decade support the hypothesis that *experimental hypertension* in its early stage is due to the release of a pressor agent from the kidney. Attention has been focused on renin and on its ability to form hypertensin enzymically from a plasma globulin. The existence of amino acid decarboxylases in the kidney and their ability to form pressor amines from aromatic amino acids has also been recognized. It is possible that such pressor amines contribute toward the elevation of blood pressure in experimental hypertension. Once chronic hypertension has become established, renin could not be found in the blood of hypertensive animals, and the experimental data point to the conclusion that the mechanism maintaining high blood pressure has changed in the second phase of the disease to one of sympathetic activity.

Renin and hypertensin have not been found in the blood of patients suffering from *essential hypertension*. However, hypertension in man is rarely diagnosed and classified in the initial stage of the disease. In view of the results with late hypertension in animals, the absence of renin in the blood of patients with well-established hypertension is not surprising. It is quite likely that these patients were already in the second phase of the disease, neurogenic hypertension, when their plasma was examined for the presence of renin and hypertensin. Failure to find these substances does not disprove the renal origin of essential hypertension.

The present knowledge of the sequence of events is undoubtedly far from complete. Much investigative work is necessary before the real significance of kidney enzymes for the production of human essential hypertension can be considered clearly established. Whether pathological changes occur in the human body in the early stage of the disease, comparable in their effect on kidney metabolism to the application of a Goldblatt clamp in animals, is a question of fundamental importance. In experimental hypertension, an understanding of the transition from a renal pressor mechanism to a sympathetically mediated process, is a necessary prerequisite for a rational therapeutic approach.

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# RECENT PROGRESS IN INDUSTRIAL FERMENTATION

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## I. Introduction

### A. PURPOSE AND SCOPE OF REVIEW

Review of progress in any field of science in these days of rapid advance calls for limitation since it is not possible to cover developments adequately even in review unless the field is a restricted one. The subject of industrial fermentations is certainly not in the latter category. Therefore this review will be limited to the developments in alcoholic fermentation, both ethyl and butyl, and to the 2,3-butylene glycol fermentation which was extensively investigated during the war period. Most of the references and discussion will refer to the work done since 1940. It is also proposed to point out the broad course of general industrial advance in the selected fermentations rather than to emphasize the theoretical side of the subject. As a matter of fact, during this particular period, efforts of research workers and manufacturers alike were directed toward production and there was little time for theoretical work. The work reviewed will be limited largely to that done in this country. Foreign developments were disrupted by the war and records of such progress as was made are just beginning to be available as contacts with the European countries are re-established. Such results, therefore, would be more appropriately summarized elsewhere. Industrial fermentations have many important aspects other than the purely bacteriological and the review will therefore include references to recovery of products, disposal of stillage, and economic considerations, all of which are part of a well-rounded view of the subject.

### B. RELATION TO WAR EFFORT

Since the period covered by the review is the war period, it is in order to treat the subject in its relation to the national war effort. In industrial fermentation as in other activities, the war gave a great stimulus to practical research and manufacturing activity. Certain results were obtained in a short time which would have taken many years under ordinary conditions. Government institutions played an important part in assembling and correlating information and in getting interested persons together for the purpose of pooling information. The technical data needed for some of the wartime production tasks were available and more or less generally known. In other cases, extensive research and pilot plant development had to be instituted in order to establish a basis for actual production. For industrial fermentation projects, these ends were greatly aided by experimental work at the Northern Regional Research Laboratory and by the

initiative of its staff in calling conferences for the guidance of those working in the industry and research institutions. At these meetings there was an interchange of information which greatly accelerated progress. The Forest Products group also gave substantial aid in developing certain aspects of technology connected with the utilization of wood for the production of ethyl alcohol and other products. Valuable contributions were made through the activity of the Canadian Research Council, and in addition many industrial and university groups made essential contributions to the developments here reviewed. Thus, the wartime fermentation developments have more than technical value and a review would fall short of its purpose if it failed to leave the reader with the feeling that these fermentation advances represent the result of a high degree of co-operative effort. This fact gives them a historical interest which they have in common with many other wartime developments.

#### C. FERMENTATION AS AN INDUSTRIAL PROBLEM

It may clarify the treatment of the industrial developments which are to be reviewed to call attention at this point to a fact not always considered, or at least not ordinarily given its proper weight in discussions of manufacture by fermentation means. This is that the fermentation itself is often the least troublesome factor in the over-all problem. It is not always realized by investigators removed from the industrial scene that such is the case, and as a result the fermentation process gets more than its share of attention and relatively little effort is expended on other aspects absolutely essential to the establishment of an industry. One of the beneficial effects of the wartime activity lay in the fact that it emphasized the importance of recovery of products, waste disposal, and economic aspects to a large group whose interest had been largely in fermentation as a bacteriological and biochemical process. Here, as in other broad developments, it is necessary to bring widely separated abilities to bear on the project in order to establish it on a sound basis. The manner in which the interested groups responded to this need is well illustrated by co-operative work on the 2,3-butylene glycol project which is taken up on pages 601-611.

#### D. PATENTS AS A SOURCE OF INFORMATION

It is not always as easy to follow industrial development as to follow purely scientific progress in any field. Information on such developments, when it becomes of real significance, has a tendency to "go underground." In many cases, the first information available on important findings is in the

form of a patent. Unless the research has been covered otherwise by publications, this delays general understanding of the advances in the field. It must therefore be borne in mind that descriptions of industrial progress based only on the patents are likely to need considerable correction. War-time developments were less subject to secrecy than those of peacetime because pressure of the national emergency made exchange of information mandatory. There are certain portions of the matter covered in this review, however, on which little beside patent information is available, and in these cases it will be necessary to ask the indulgence of readers in possession of special information if the matter presented seems inadequate.

## II. Butanol-Acetone Fermentation

### A. INDUSTRIAL IMPORTANCE

The butanol-acetone fermentation has been such an important commercial source of butanol since the first world war that considerable patent activity has been directed toward protection of fermentation processes and certain features accessory to the fermentation process. Early production by fermentation was entirely from grains, and for many years sugar-containing raw materials were not used in manufacture of solvents by fermentation. However, the fact that by-product molasses is the lowest cost fermentation raw material under ordinary circumstances has stood as a challenge to producers of solvents, and it was inevitable that the technical problems relating to its use would be solved. Figure 1 shows the unloading of a tanker of West Indian molasses at an alcohol plant and illustrates the ease with which this raw material is handled. Many of the patents and process descriptions deal with the use of molasses, especially cane molasses, while others relate to sterilization of plant equipment and still others to control of the bacterial metabolism and protection of the organisms against phage.

### B. PECULIARITIES OF THE BUTANOL FERMENTATION AS THEY AFFECT ITS INDUSTRIAL DEVELOPMENT

Butanol fermentation has until recent years stood alone among the commercial fermentations in the respect that it does not contain what might be called factors of safety. Some industrial fermentations, especially the older ones, do contain such factors and they can, therefore, be operated much more readily than the butanol fermentation. In the latter, the necessary conditions for the organism concerned include, for instance, a

pH value around 6.0. This renders the medium an excellent one for acid-forming organisms in general. Such organisms, introduced into the complicated valve and piping systems of an industrial plant, will overgrow the solvent-producing forms, since the invaders are more tolerant of acid than the solvent producers, with the result that the whole course of the fermentation changes and there is complete loss of the material involved. Ethyl

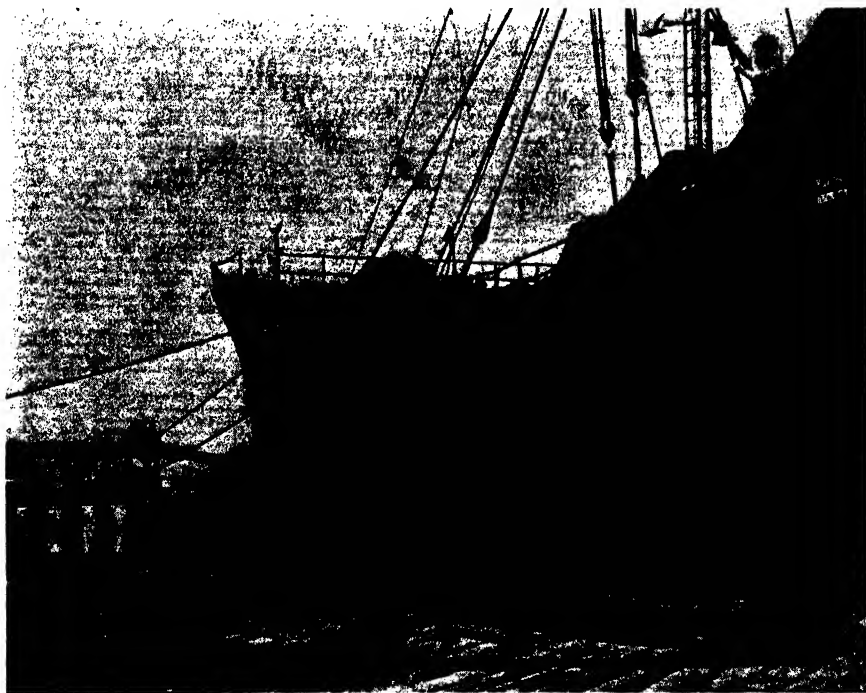


Fig. 1. Unloading a tanker of molasses for fermentation use (courtesy U. S. Industrial Chemicals, Inc.).

alcohol fermentations give low yields under unfavorable conditions, but do not fail completely as in the case with the biologically unstable butyl alcohol fermentation. Other industrial fermentations, such as the acetic acid, for instance, are inherently stable. Knowledge of the technique of preventing contamination of large operations is thus one of the principal assets of any manufacturer of solvents, and little is written on this subject. There are obviously many aspects to preventing contamination in a plant. It is

necessary for instance to have well-trained personnel, good housekeeping, and, above all, a *vigorous and rapid* fermentation. The latter requirement explains the numerous patented processes which have been built around special strains of solvent-producing organisms. Each of these organisms has specific requirements differing slightly from those of other strains and various workers have determined empirically suitable nutrient solutions and processing conditions to give the vigorous fermentation which is a *sine qua non* for commercial operation. A further observation should be made concerning the use of sugar-bearing raw materials, especially molasses. As noted above, grain was for many years the only material used for butanol-acetone fermentations. The fact that molasses was not used is difficult to explain in view of its lower cost. In all probability this delay in learning to use molasses was due to failure to recognize the fact that certain strains of solvent-producing bacteria work well on starch while others work well on sugar. It is very difficult to get the starch types to ferment sugar satisfactorily and vice versa. The use of molasses, therefore, called first for isolation from nature of new strains adapted to utilize sugar, and second for setting up a growth medium suitable for these organisms. This is reflected in many of the recent patents where the description of a new organism is combined with a process for its commercial use.

### C. PROCESSES AND PROCESS PATENTS

In a patent taken out by Muller (90) there is described an organism said to ferment sugar up to 10% concentration in a medium containing only molasses, an ammonium salt, calcium carbonate, and a small percentage of vegetable protein. This is a very high percentage of sugar to ferment to completion. Stiles and Pruess (125) describe a process involving use of stillage from an ethyl alcohol fermentation as an ingredient in a butyl alcohol mash, and stillage from the butyl alcohol mash as an ingredient in ethyl alcohol mash. This is to be repeated for a number of cycles, and then discontinued just short of the point at which inhibition of the fermentation sets in. Since the use of stillage is important in plant operation as a means of conserving water and, where evaporation is necessary, of economizing steam, this suggestion is an interesting one. Loughlin (83) describes a process for production of butyl and isopropyl alcohols by a sugar type organism, using a degraded protein as a supplement. Lagotkin (72) describes a continuous system for butyl alcohol. In view of the ease of contaminating butyl alcohol fermentations, continuous operation would be hazardous. Arroyo (5) outlines a molasses process in which the sucrose is

inverted by acid, and an "activating agent"—clay or some type of carbon—is added to the molasses. This process is carried out with a strain isolated by Arroyo from the roots of a variety of sugar cane. Legg and Stiles (77) describe a process control procedure which involves buffering the mash with organic acid salts of alkaline earth metals. Woodruff, Stiles, and Legg (148) propose to use a sugar-fermenting organism on a molasses mash containing digested protein with chalk as a neutralizer. Weizmann (143) recommends the use of peanut cake with molasses. Ierusalimskiĭ and Semenova (62) describe a process in which a mixture of grain and beet molasses is fermented. This requires a special procedure in that the beet molasses is added after the fermentation is partly finished. The attempt to ferment both starch and sugar with the same organism very probably complicates the process for the reason previously noted. Weizmann (144) proposes a method by which the organisms are started on rice bran mash to which molasses is added after an initial multiplication stage and digestion of the bran protein to produce amino acids. Hall (48) uses a new strain, *Bacillus butacene*, isolated from humus which ferments sugary or starch mashes supplemented with animal or vegetable protein.

None of the published data on the butyl alcohol fermentation either in the scientific literature or in the form of patents indicates any fundamental improvement in the fermentation itself. It would be useful to the industry if the process could be safeguarded against contamination by some means so that minor plant-operating irregularities would have less effect on the fermentations. It would also be helpful if a way could be found to ferment more concentrated solutions. The hazards of this type of fermentation have been a factor in limiting it to a few large companies in this country. These organizations have obtained the experience necessary for running the process by observations of actual plant operations under various conditions and have at the same time built up the trained personnel required. If the process could be made more certain by developing inherent factors of protection against invading organisms, it might be more widely used and there would be a better chance of utilizing agricultural wastes or special crops.

#### D. PROTECTION OF BUTANOL-ACETONE FERMENTATION AGAINST PHAGE

One of the serious and well-known operational difficulties in the butyl alcohol fermentation is its susceptibility to "sluggishness" or bacteriophage. In the former case, the process starts as usual with the organisms multi-



plying in the mash and building up acid in characteristic fashion. If the fermentation is normal, these acids then disappear with the formation of solvents so that the fermentation finishes with low acid content and low residual sugars. However, when the sluggish type of fermentation is encountered, the acidity peak is reached but the organisms do not continue the process which results in solvent formation, and fermentation finishes with a high residual sugar. The organisms found in these fermentations have an etched appearance, indicative of partial lysis by a phage-like body. Legg and Walton (78) have described a procedure for immunizing against this condition. Also McCoy, McDaniel, and Sylvester (84) described an outbreak of true bacteriophage in a newly opened plant. These authors made a thorough study of the phage strains, including serological tests, and described the immunizing methods used to overcome them. Bacteriophage in a butyl alcohol plant can become quite serious under certain conditions, so serious in fact that a plant may have to shut down. The paper by McCoy and co-workers is especially interesting as a guide to handling phage outbreaks in butyl alcohol fermentation.

#### E. BUTANOL FERMENTATIONS OF MATERIALS OTHER THAN MOLASSES AND GRAINS

During the last four years, various publications have appeared proposing to ferment industrial and agricultural wastes for the production of butanol and acetone. While any sugar-containing material has potential use as a raw material for this fermentation, the sensitive nature of the organism and in particular the extreme susceptibility of the fermentation to contamination make commercial operation with certain raw materials difficult. Sulfite liquor, for instance, which can be readily fermented by yeast after a simple treatment, requires special purification for butyl alcohol fermentation. By way of giving a general survey of fermentations proposed in recent years, the following references may be cited.

##### *1. Farm Products and Miscellaneous Materials*

Legg and Stiles (76) describe a method for the fermentation of hydrol, the molasses-like by-product from the manufacture of corn sugar. This material needs ammonia nitrogen and organic materials, the latter being supplied by stillage from grain fermentation. Citrus and apple wastes can be fermented without supplementation according to Rosenfeld (108). The fermentation of cassava was studied by Banzon, Fulmer, and Underkofler (10). This material must be supplemented, and the authors suggest

corn, corn gluten meal, soybeans, and shrimp powder as suitable supplements. The last-named material was considered because of its ready availability in the Philippines where the cassava is also available. A new organism is proposed by Jean (65) for fermenting ground sterilized garbage. He claims about ten gallons of mixed solvents per ton of garbage. The use of sweet potatoes or waste from sweet potato starch manufacture is described by Doi and Yamada (35) and Brown and Brinson (22). Sweet potato mashes may be fermented without supplementation although supplements of cotton seed meal, soybean cake, or rice bran pay for themselves in higher solvent yields. Wendland, Fulmer, and Underkofler (145) found that Jerusalem artichokes could be used provided they were hydrolyzed first by heating with mineral acids. Supplementation was necessary, soybean meal giving the best results.

### 2. Sulfite Liquor as a Raw Material

Waste sulfite liquor which is produced in enormous quantities as a by-product in paper making contains fermentable sugars and is always considered a potential raw material for fermentation processes. Wiley, Johnson, McCoy, and Peterson (146) worked on the butyl alcohol fermentation of this substance. Since the liquor is toxic, these workers adopted the logical approach of trying a number of strains of bacteria on liquor purified of many of the poisonous ingredients. Sulfur dioxide was precipitated with lime, which was also used to throw down lignin. The excess calcium was removed by addition of sodium sulfate, the pH was adjusted to 5.8, and ammonium orthophosphate,  $(\text{NH}_4)_2\text{HPO}_4$ , was added as a nutrient with calcium carbonate to regulate acidity during fermentation. By using such a solution with the best strain, *Clostridium butylicum* (Fitz), results comparable to those from molasses were obtained so far as efficiency of sugar utilization was concerned. Rieche *et al.* (104–106) have taken out patents in Germany on a process and on special process features for butanol production from sulfite waste. Grondal and Berger (47) have recently announced a comprehensive processing scheme for waste sulfite liquors which produces butanol as one step. If this process lives up to the hopes of the inventors, it might become important as a source of butanol.

### 3. Economic Considerations Affecting Use of Special Materials

It should be noted that all these authors stress the fermentation aspect of the problem. While this is relatively more important in the case of the butanol fermentation than in other fermentations, solvent production from

most of these materials would involve plant and economic problems of considerable magnitude. The use of fruit wastes or farm products involves a collection and preservation problem which would add greatly to the cost of production, and proposals to use such materials are incomplete without some economic analysis. The use of sulfite liquor requires a considerable investment in stills, condensers, tanks, etc. made of corrosion-resistant metals such as stainless steel; plant construction costs as well as the low sugar content of the liquor would have to be considered in the fermentation of this material. Establishment of a fermentation industry based on the sugars and starch sources noted above depends, therefore, on the solution of the accessory problems as well as on learning how to carry out the fermentation itself.

#### F. MAINTENANCE OF STERILITY

The technic of maintaining sterility in the necessarily complicated piping and tank system of an operating, large scale plant is one of the principal problems of the butanol fermentation. Individual manufacturers have solved this by taking known precautions against contamination such as sterilization by live steam, use of antiseptics where possible, and attention to many details connected with plant design, operation, and personnel. Figure 2 shows a mash cooler designed for operation under aseptic conditions. The knowledge of this technic is an extremely valuable stock-in-trade and as previously noted, very little has been written on this aspect of the butanol fermentation, important though it is. However, among the patents taken out during the period covered by this review are two by Boeckeler and Bogart (19,20) and one by Baldwin (7)—all three on aids to the maintenance of sterility. The Boeckeler-Bogart patents describe a system operated by steam injectors which circulates gases for stirring the mash and also introduces steam and sterile air, or sterile air alone into a fermenting vessel. These operations are accomplished with a minimum of apparatus, although it is necessary to complicate the fermentation system somewhat by the introduction of pipes and spargers and by the use of a cooler connected into the fermentation system. It is claimed also that stirring the mash during certain fermentation stages permits intimate contact between the calcium carbonate, used as a neutralizer in molasses butanol fermentations, and the fermenting solution, thus saving calcium carbonate and assisting the organisms to pass through the acid "break."

Baldwin (7) has patented a system for inoculating the starter tank in the series of seeds used in the butanol fermentation. Prevention of con-

tamination at the time of this primary inoculation is of great importance, since acid-forming organisms introduced here will multiply during the seed stages and may build up sufficiently to lower the yield in the final fermenter or even to stop fermentation in the last stage. The system described by Baldwin safeguards the mechanical steps of transfer of inoculum to prevent



Fig. 2. Cooler designed for large-scale pure culture fermentation operation (courtesy U. S. Industrial Chemicals, Inc.).

entrance into the plant system of organisms other than those in the inoculating flask.

These improvements are ingenious and have to be evaluated by balancing their value as mechanical aids to operation against the bacteriological hazard introduced by any complication of the piping or tanks immediately involved in the fermentation.

As may be seen from Figure 3, the piping necessary for the operations

of cleaning, steaming, and filling the large fermenters of a butanol plant involves numerous valves and flanged connections. These are protected from infection by being kept under steam pressure when not in use. In general, maximum simplification of all the fermentation accessories is required for safe operation.



Fig. 3. Fermenters equipped for pure culture butanol fermentation (*courtesy U. S. Industrial Chemicals, Inc.*).

Shaposhnikov and Bekhtereva (118) suggest the continuous removal of solvents as they are formed by extracting the mash with a solvent immiscible with water, as, for instance, castor oil. This is an example of an ingenious process which might have some theoretical merit, but which would be extremely difficult to carry out on a large scale due to the introduction into the fermenting system of complicated apparatus not easily kept clean and sterile.

G. VITAMIN-RICH BY-PRODUCTS FROM MOLASSES AND GRAIN  
STILLAGE AND FROM WHEY, WITH SPECIAL REFERENCE  
TO BUTANOL FERMENTATION

*1. Historical*

The disposal of by-products and of waste is an ever-present problem in any manufacturing operation and may, indeed, become so acute that the survival of an industry depends upon a satisfactory answer to the economic and technical questions arising in this connection. The fermentation industries have struggled with such by-product and waste disposal problems for many years and have attained some success in solving them. Certain by-products such as distiller's grains have definite value in animal feeds. In recent years, the regulations against stream pollution have forced complete recovery of the residues from grain distilleries in many locations and this has resulted in the marketing for feed use of dried residues made from thin liquors after distillation for ethyl alcohol production. Fortunately, advances in knowledge of vitamins have supported the feeding of these distillery by-products and enabled them to be used in such a manner as to take advantage of their good properties. Efforts to solve the disposal problem especially in the case of distillers using molasses have led to a new commercial source of vitamins in the establishment of which butanol fermentation has had an important part. The present importance of this source justifies a brief review of the history of the development which began in an obscure fashion with little indication as to where it would ultimately lead.

The molasses distillers have been in a less favorable position to benefit from the sale of distillery by-products than the grain distillers and, as a consequence, it has always been considered the most economical procedure to dump the waste stillage if possible. This was due to the fact that stillage from molasses ethyl alcohol plants had not, until a few years ago, been known to contain values over and above its fertilizer value, and the cost of obtaining the fertilizer elements as such was greater than their market price. Nevertheless, molasses distillers, in some cases, had to process their wastes in order to dispose of them without causing stream pollution even though the processing was done at a loss. This stimulated research on ways to realize on the values present in the stillage from molasses fermentation plants. Evaporation and incineration of the concentrates produce a salable fertilizer rich in potash but not sufficiently valuable to pay for the processing. In this portion of the review it is desired to outline in a general

way the recent growth of by-product utilization, particularly from molasses stillage. Owing to the extension of knowledge of vitamin requirements in feedstuffs this utilization has had a rapid expansion and its ramifications are such that it is not possible to treat the subject in an entirely logical manner. For instance, processes are now in operation in which whey is first fermented for solvent production and the stillage becomes an important source of riboflavin. It should be borne in mind that these developments had their beginnings in the necessity to dispose of by-products economically and to the best advantage. Since these processes have considerable commercial importance, their development has to be traced largely through the patents taken out to cover them rather than in journal publications. Most of this development has taken place in the last five or six years, although its beginnings go much further back.

## *2. Process Development and Patents on Use of the Butanol-Acetone Organism*

In the case of molasses stillages, the reduction to a dry state involves first evaporation to a syrup of about 50% solids content and second drying. Both evaporation and drying involve problems of considerable magnitude. In 1927, a patent was issued to Dickerson (34) on a method of spray-drying molasses or molasses stillage for use as a fertilizer. This process never reached the stage of commercial application, but the patent is interesting in showing how to produce a spray-dried product from the residue of a molasses distillery. In 1935, Lewis (81) obtained a similar patent on the spray-drying of molasses or molasses residues, and in 1938 he obtained a second patent (82) in which he covered the process more completely. In both of these, the procedure is designed to avoid injury to the food values, since the spray-dried materials were destined for use in animal feeds. Although no publication of the vitamin value was made, investigations of the vitamin content of this product were made very early in its development (in 1929) and it was shown by rat tests that vitamin B<sub>2</sub> was present in significant amounts. This led to the sale of the spray-dried material as a carrier of vitamin B<sub>2</sub>, or riboflavin. A considerable business has been built up on this product and similar ones by one of the large producers of alcohol from molasses. Initially, the product, which is a particularly useful supplement to poultry feed, was made from molasses ethyl alcohol stillage. The nutritional phases of this product are covered by Bird and Groschke (15) and Bird and Mattingly (16).

In 1938 an article by Yamasaki (150) stated that riboflavin was produced by the butyl alcohol bacteria in large amounts. This investigator applied early in 1938 for a Japanese patent and later in the same year for a U. S. patent on this method of producing the vitamin. U. S. patent 2,297,671 granted Yamasaki in 1942 covers the process for starchy mash, sugary mash, and whey. Miner (87) obtained a patent in 1940 covering the use of molasses and other raw materials. Yamasaki (151) published further data in 1941 on the formation of flavin and called attention to the fact that iron is a specific poison for the process. In 1943, Arzberger (6) was granted a U. S. patent on a process to prevent the harmful action of iron and other toxic metals by purification of the mash and by growth in aluminum vessels. It is thus apparent that investigations were being made concurrently in the United States and in Japan. Also, the known necessity for riboflavin in poultry feeds and the fact that a growing business was being established in fermentation by-products specifically rich in riboflavin, greatly stimulated activity in this field. A number of patents were granted to cover process steps including both production and recovery (31,59,68,85,140). Not only were the usual butyl fermentations of starchy and sugary mash included in this attempt to cover the field by patents, but the use of whey as a fermentation medium for the production of solvents and the recovery of a product rich in riboflavin was also covered (86). The last-named takes into account the effect of iron on the production of riboflavin by solvent-producing organisms. Further commercial developments include those of Legg and Beesch (75) who claim that the addition of sulfite radical increases the riboflavin content of a butanol fermentation. Also, Hines (60) describes a process in which one organism is used to make possible recovery of riboflavin produced by another.

An interesting study of the relation of iron to riboflavin production was made by Leviton (80). He reports the vitamin very sensitive to hydrogen peroxide if ferrous iron is also present. In pure solutions, the peroxide is without effect but at a concentration between 0.018 and 0.036 milligram atoms of ferrous iron per liter, there is an abrupt increase in the rate of decomposition of riboflavin. When *Clostridium acetobutylicum* synthesizes riboflavin, the vitamin production is drastically reduced at this concentration of iron. This suggests that the effect of iron on the riboflavin synthesis acts through a peroxide mechanism, and Leviton gives evidence that this is true. Significant increases in yield were obtained by the use of sodium hydrosulfite and traces of crystalline catalase. The use of sulfite by Legg and Beesch is thus explained by Leviton's work.



### 3. Use of Organisms Other Than Solvent Formers to Produce Riboflavin

The commercial value of riboflavin as a supplement to poultry feeds has stimulated the search for organisms other than the solvent producers to give the vitamin as a main product rather than as a by-product. The possibility of obtaining higher yields is attractive, and an even more pressing reason for such efforts lies in the sensitivity to iron of riboflavin production by the butanol-acetone organisms. The existence of a number of patented processes to avoid this difficulty shows its importance, which is indeed obvious due to the fact that fermentation tanks are preferably made of steel, and iron in one form or another is present in most commercial raw materials. The use of special metals requires plant construction which may be noneconomic or even impossible under present conditions. Among investigators suggesting organisms other than the solvent-producing bacteria is Burkholder (23,24), who reported on a yeast, *Candida guilliermondia*, which gave high riboflavin production in a special medium. In 1944 he obtained a patent (25) on a process using this yeast in a synthetic medium to which cyanide was added at a certain stage of the fermentation. Unfortunately, this fermentation is also susceptible to inhibition by iron. The amount of the metal required for maximum growth is much greater than the amount which gives the optimum production of riboflavin. This relation of vitamin production to iron is treated in a paper by Tanner, Vojnovich, and Van Lanen (128). Rudert (109) has patented a process for the production of riboflavin by the use of the fungus *Eremothecium ashbyii*. This patent covers the composition of the medium and physical conditions of fermentation necessary to produce the vitamin in quantity. The culture solution is unique in that it contains little or no carbohydrate. Lipides such as corn oil, olive oil, peanut oil, etc., together with proteins and nutrient salts, make up the medium. The fermentation may be run as a deep vat type of fermentation with or without mechanical agitation. Recovery of the vitamin may be accomplished by evaporation to form a concentrate and finally by complete drying using accepted methods, such as sprays, drums, etc. No specific mention is made of iron sensitivity in describing this fermentation although the amount of iron specified is low (10 p.p.m.). Rudert's process thus completes a cycle of development which begins with the discovery of a valuable food element in a fermentation by-product and ends with the production of this food element as the principal product of an entirely different fermentation using a special medium and organism. The manufacture of vitamins by fermentation is very active at this time and will, no doubt, be exploited increasingly in the future. As

the above brief summary indicates, it had its beginnings in the necessity to dispose of a fermentation by-product.

### III. Ethyl Alcohol Fermentation

#### A. INTRODUCTORY

The part played in the war by fermentation alcohol and the industry behind its production has never been publicized as were many other aspects of the war effort. Although the accomplishments were not made in a spectacular manner, their importance is obvious when it is considered that the peacetime alcohol production of the country was quadrupled during the war and that about half of the increased production went into the synthetic rubber program. The greater part of this alcohol was supplied by the fermentation industry, with very little new plant construction and in the face of complicated supply problems. Yeast fermentation of molasses had to compete with the use of molasses as an ingredient of mixed feed for animals and even with the use of sugar for human food. The industry and responsible government officials had to reckon with the impact of submarine warfare on transport of molasses from the West Indies. Agricultural, industrial, and regional pressures arose for greater use of substitute raw materials ranging from corn and wheat to wood waste and sweet potatoes. The alcohol producer often was obliged to use grain when animal feedstuffs were in short supply. In addition to these factors, the alcohol program was affected by commodities superficially unrelated to alcohol manufacture but required for the vitally important synthetic rubber production.

Means of meeting the complicated and rapidly changing requirements of the war program were worked out co-operatively by the government, the industry, and various research groups in university or government laboratories. (For a summarized description of government-sponsored plants see reference 136a.) The technic of production from molasses was well known and required no investigation. The surplus of wheat which developed during the war had to be used in part for alcohol production and although this was an unfamiliar raw material it was used successfully for fermentation after a brief period of experimentation. For use in case of necessity, it was desirable to have available, technics for fermentation of processed wood wastes, sulfite liquor, and certain farm products. The required methods were outlined by preliminary experiments and pilot plant work, and in some cases, operations on a commercial scale were initiated. In order to take

care of possible shortages of malt for the conversion of grains or other starchy raw materials a substitute converting agent, mold bran, was brought to the point where it would be produced on a large scale. These and other lines of activity are reviewed in the sections immediately following.

## B. ALCOHOL FROM WOOD

### *1. Acid-Digested Wood as a Source of Sugar*

The production of alcohol from wood and wood wastes has a long history both on the investigative and industrial side. It is not the purpose of this review to go into details of this history except in so far as is necessary to explain recent developments. The idea of saccharifying wood and fermenting the sugar so produced was conceived over a century ago, and sulfite liquor fermentation was patented in 1878. Since that time, processes have been improved and advances made as the economic situation permitted. A great part of this development has been European for reasons that are obvious. Among these are low labor cost and the shortage in Europe of inexpensive raw materials for alcohol production, which stimulates search for sources of fermentable sugars. Also, in the early years of the century, the technological background for work of this type was more highly developed in Europe than elsewhere, with the Scandinavian countries and Germany especially responsible for noteworthy progress in this field. This backlog of information obtained on the continent has been of the greatest importance in developments in the United States.

Prior to the second world war, attempts had been made to establish an alcohol industry in the United States both on the basis of wood saccharification and on the basis of sulfite liquor fermentation. One of the first of these attempts grew out of the work of Classen (28) and was a serious attempt to commercialize his process beginning with pilot plant runs and ending with the establishment of a commercial unit at Hattiesburg, Mississippi. The process involved pressure saccharification of the wood by means of sulfurous acid and heat in a large lead-lined digester followed by extraction of the sugar, elimination of sulfur dioxide and other impurities from the liquors, and fermentation of the sugars by yeast. Eighteen to twenty gallons of absolute alcohol were claimed per ton of wood waste processed. The operation of the Classen process ran into major mechanical difficulties, however, and the Hattiesburg plant never ran successfully.

The next attempt to set up a workable scheme for processing wood was made by two chemical engineers who had been associated with Classen.

They made modifications in the digestion process and equipment which gave more rapid digestion and less mechanical difficulty. Their efforts ultimately led to the erection of a plant at Georgetown, South Carolina. Among the changes made was the substitution of sulfuric acid for sulfurous acid. This plant ran successfully for a period prior to and during World War I. It is described by Demuth (32). A plant was erected at Fullerton, Louisiana, to use essentially the same process as the Georgetown plant. This plant operated during World War I and for some time thereafter on sawmill waste. The process involved digestion of the finely divided wood in tile-lined spherical digesters of about six tons capacity. These were charged with wood and the required amount of sulfuric acid, closed, and steam was introduced directly to bring the pressure to 120 lb. After fifteen minutes, the digester was blown down to atmospheric pressure and the digested wood conveyed to a battery of extractors similar to those used in the beet sugar industry. Countercurrent hot water extraction gave a solution containing 6–9% reducing sugar. This was neutralized, settled, cooled, and the clear liquor was fermented by yeast. The wood residues were burned to produce steam. The plant produced 28 gal. of 95% alcohol per ton of wood processed, and the alcohol was of high purity.

It is instructive to consider some of the factors leading to the closing down of the Georgetown and Fullerton plants. In neither case was the shutdown caused by the inoperability of the process as was true of the Clausen plant. These plants, operating on sawmill wastes, become useless if the waste has to be transported long distances. Lumber operations follow the forests and have to be moved periodically as timber is cut over. The same requirement applies to alcohol plants attached to lumbering operations. The rate of cutover was so rapid at the Fullerton plant sawmill that it forced a curtailment of operations, which with competition from the low cost molasses process eventually led to the closing of these plants.

### *2. The Sulfite Liquor Process*

The sulfite process has also been operated in this country following the lead of developments in the Scandinavian countries. Two experimental plants were installed in Sweden in 1907, and United States interest in the development was aroused at once, although no plant was actually built here until 1913, when one was erected at Mechanicsville, Virginia. This plant was put into operation by Ekstrom, who developed the Swedish process. It was a modern installation with a capacity of 6500 gal. of alcohol per day. The fermentation tanks were made of steel. Liquor from the

digesters, where the wood is treated with sulfurous acid, was received in treatment tanks. There it was limed and allowed to settle, and the clear portion was decanted for use in the fermenters. Settled bottom yeast from previous fermentations was left in the fermenter as seed for fresh mash. This method was proved to be practically as good as preparing fresh yeast for each run. This plant was operated over a period of 25 years, although not continuously. During a portion of this period of operation, molasses was used with the sulfite liquor. It was finally concluded that unless the market price for alcohol was fifty cents per gallon or higher, the plant could not be operated at a profit.

As a concluding statement to this survey one cannot do better than to quote from the excellent summary by Sherrard and Kressman (119), from which most of the above information was taken:

"One is forced to the conclusion...that the technology involved in the conversion of wood waste and sulfite liquor is fairly complete. Undoubtedly ways and means will be found to increase slightly the yields now obtainable and by-products will be more efficiently used; but the fact remains that the commercialization of either of the processes will ultimately be decided largely by the prevailing economic situation."

### *3. Wartime Developments—Wood Digestion Process*

This was the situation in the United States at the beginning of World War II. As is well known, the requirement for alcohol during the war was enormously increased by the needs of the rubber program. Instead of the annual 150,000,000 gal. of peace time use, over 600,000,000 gal. were needed, most of which had to come from fermentation. The usual source of fermentation alcohol—blackstrap molasses—was not available in sufficient quantity to take care of this great increase and it was necessary to turn to grain as a raw material. The government agencies charged with the task of providing for the immediate needs of the rubber program and for insurance against possible shortages in case the war was prolonged realized that plans had to be made to utilize other raw materials, since molasses was hard to get due to shipping conditions during the war, and since grain might well become scarce due to food needs.

Consideration of these facts naturally led to plans to use saccharified wood waste and sulfite liquor as sources of fermentable sugar. Large potential supplies of these were available, and the economic factors which affect their use in peacetime did not enter into the picture as serious deterrents in the emergency. Faith and Hall (41) summarize the wood hydrolysis process of Scholler as modified by work in the United States.

In another paper, Faith (40) also outlines the manner in which the government functioned to insure production of alcohol from saccharified wood in the United States if and when it became necessary. This paper gives a description of the Scholler process as practiced in Germany and calls attention to some of the difficulties encountered there. In order to have a sound basis for future developments the O. P. R. D. of the War Production Board made it possible for pilot experiments to be carried out at Marquette, Michigan, along with parallel work at the Forest Products Laboratory. Faith calls attention to some of the factors that limit the use of wood waste even in wartime. Such things as type of wood available may make large differences in yield. Also, it is necessary to insure an adequate supply of wood, as hauling costs may become prohibitively high if the alcohol plant is unable to supply its needs from nearby mills. He also stresses the fact that a hydrolysis plant is the most costly type of alcohol plant to build because the inherent nature of the process requires acid-resistant equipment. On the other hand, the discovery of uses for lignin might alter the whole economic picture on this type of alcohol production. The work described by Faith and Hall (41) was the basis for a government-financed plant at Eugene, Oregon, which did not, however, come into production during the war. Since improvements in the chemical processing necessary before fermentation are an integral part of the picture in the use of wood, it is desirable to include them in this summary.

#### **American Work on the Digestion Process—The Hydrolysis Step.**

As a result of studies at the Forest Products Laboratory the Scholler process was modified to make possible definite operation improvements. Harris, Beglinger, Hajny, and Sherrard (52) report in some detail on pilot plant experiments carried out there. These workers used a stationary digester charged with about 375 lb. of dry wood. Dilute sulfuric acid was the hydrolyzing agent and heat was supplied by direct steam. In order to prevent loss of sugar by breakdown at the high temperature necessary for digestion, the acid treatment was made in a number of steps or cycles, sugar solution being drained from the digester at the end of each cycle. Softwoods were shown to have potential yields varying from 40–60 gal. of alcohol and hardwoods yields ranging from 33–42 gal. of alcohol per ton. The sugar content of the digester draw-off varied in such a manner as to indicate that hemicelluloses were digested first and the  $\alpha$ -cellulose later. Studies were made of a number of the factors entering into the process. It was found that very fine sawdust did not process well, owing to the difficulty of obtaining rapid percolation. Sugar in solution retained too long

in the reaction chamber is broken down, and, in addition to the loss of sugar, the reaction time is lengthened. High acid concentration was found to give better digestion than lower concentration, since it is especially necessary to keep the acid concentration high in the first stages of the process. Temperature is made low on the first cycles and higher as the digestion approaches completion, while the acidity is varied in the opposite direction, to minimize sugar breakdown. The ethyl alcohol fermentation of the recovered sugar liquors was carried out under conditions not possible in practice, but it was explained that this was necessary in order to evaluate the process. Liquors were first neutralized and then inoculated with an actively fermenting seed culture, equivalent in volume to 5% of the final fermentation. The final fermentation contained relatively large amounts of ammonium salts and other nutrients, a total of about 0.7% in a solution containing from 3-6% of reducing sugar, amounts impractical for commercial operation. In addition to yeast fermentation, the digest liquor was fermented for butanol-acetone production. In this fermentation the furfural content of the liquors was critical but normal yields of 27-29% on the sugar could be obtained. *Aerobacter aerogenes* was also used to produce 2,3-butylene glycol with combined glycol and acetoin production equivalent to 36% of the sugar charged to the process.

The above work has been considered in some detail since it was well planned and carried out, and resulted in cutting the time to about one-half to one-fourth of that formerly necessary in the Scholler process. This achievement brings closer the practicability of wood digestion as a source of fermentable sugars.

**Hydrolysis of Wastes Other Than Wood.** Dunning and Lathrop (38) investigated the acid hydrolysis of agricultural wastes other than wood. Their experiments were made on five materials available in quantity, corncobs, sugar cane bagasses, flax shives, oat hulls, and cottonseed hulls, but the work reported deals entirely with corncobs. The opening paragraphs of their report contain some interesting comments on the problem of using agricultural wastes in a profitable manner. These workers found they could carry out the acid hydrolysis of cobs in two steps; hydrolyzing first the pentosans with dilute acid, removing the pentoses and drying the residue, and then treating with concentrated acid to hydrolyze the cellulose. By this procedure they were able to cut the amount of acid to about one-fourth that previously used. Preliminary extraction of pentoses made the cell structure more accessible to the acid hydrolysis of the second part of the process. The process was carried out for the most

part in small scale, continuously operating equipment. A countercurrent hot sulfuric acid extractor was used for the pentosan hydrolysis and by means of this, 90–95% of the pentosans of the cob were recovered in the extract as pentoses. This step could also be carried out batchwise either at atmospheric pressure and 100°C. or at elevated temperatures and pressure.

Drying of the residues from the first process step involved first pressing out the excess acid and then drying in a hot air dryer. Acid from the pentosan hydrolysis was left in the mass and this concentrated during the drying, facilitating the sulfuric hydrolysis of the cellulose. After drying, the mass was impregnated with concentrated sulfuric in a machine similar in construction to an oil expeller. By moistening the powder with acid and then subjecting it to pressure, the acid is distributed throughout the cell structure. For best dextrose yields, water was then added to bring the acid concentration down to 85% and the resulting slurry was heated to 120–130°C. for about eight minutes. This gave a solution containing 10.5% dextrose. Separation of the two types of sugar solutions permits suitable processing after neutralization. The dextrose may be fermented to alcohol and the xylose may be fermented, crystallized, or made into furfural, as desired. The raw materials for this process are thus corncobs, sulfuric acid, and lime, the products xylose, furfural, alcohol, and lignin. Considerable amounts of calcium sulfate would be obtained as a by-product. Preliminary cost calculations indicate the possibility of actually operating a process of this type, and the report promises further work on a pilot plant scale.

**Fermentation Studies.** *Production of Fodder Yeast.* Peterson, Snell, and Frazier (101) made a study of the production of fodder yeast from hydrolyzed wood as part of the general survey of the wartime possibilities of wood utilization in fermentation industries. The production of yeast from wood sugar had been carried out in Germany, and publications covering this development were noted in the report by these authors. The development of this aspect of wood fermentation had less immediate applicability to war needs than the alcohol production projects where the alcohol was urgently needed for rubber production. In the yeast growth study, the object was to produce cheap protein from substances which would otherwise go to waste and to provide insurance against a possible food protein shortage.

The authors made a thoroughgoing study of the factors involved in growing yeast in wood sugar solutions. They first considered treatments



to lessen the toxicity of the wood sugar solutions. Their most successful procedure was a modification of one described by German investigators and consisted in the addition of calcium carbonate followed by a small amount of sodium sulfite to the sugar solution, heating to boiling, cooling, and filtering. The treated solution was supplemented by small additions of urea and potassium dihydrogen phosphate, and fermentations by various yeasts were carried out on this solution after dilution to about 1% sugar. *Torula utilis* No. 3 gave good yields of yeast (about 40% dry yeast on the total sugar) and was subsequently found to be superior in its ability to adapt itself to unfavorable conditions. It was therefore used for most of the experimental work.

It was found that yields of yeast differed greatly when hydrolyzates from various species of wood were used. Spruce and southern pine fermented well, but Douglas fir was difficult to ferment. Hardwoods gave readily fermentable solutions and correspondingly higher yeast yields. The range of yield was from 30–42 lb. dry yeast per 100 lb. sugar in the hydrolyzate solutions. Other factors studied were sugar concentration (1% found optimal), aeration rate, temperature, nitrogen sources, phosphorus sources, and other factors important in the fermentation. Rather large amounts of inoculum and of air were used; both were in excess of the corresponding quantities in commercial yeast production. The authors conclude their paper with the statement that the cost of yeast produced from wood is about 25 cents per lb. of protein, adding that this does not compare favorably with other low cost protein, for instance, that from soybeans which sells at approximately 5 cents per lb.

A study of the possibility of producing yeast from the stillage of the wood sugar process for production of ethyl alcohol was made by Kurth (71). This production of yeast is desirable not only because of the value of the yeast but in order to reduce the biochemical oxygen demand of the plant wastes and thus lessen stream contamination. Kurth used yeasts able to utilize five-carbon sugars and his results indicated the possibility of producing about one ton of dry yeast per 100,000 gal. of stillage fermented. Kurth's work, which covered the laboratory aspects of the subject well, would have to be extended to answer the economic and plant operational questions involved.

*Alcohol Production from Hydrolyzates of Wood.* Leonard and Hajny (79) studied yeast fermentation of wood hydrolyzates and found it presented difficulties except under special conditions. They call attention to the fact that most hydrolysis experiments have been directed to maximum

sugar production rather than to maximum fermentability of the sugar produced. Their experiments showed improved fermentation when the hydrolyzates were treated with lime to pH 5 and then heated to 138°C. for a short time before filtering. An improvement in fermentation also resulted when reducing substances were added and among those tried were sulfites, ascorbic acid, and reduced iron. Yeasts species varied considerably and showed no acclimatization after repeated cultivation on the hydrolyzate. A *cerevisiae* type of yeast was found most suitable for the work and since yeast does not multiply readily on wood sugar hydrolyzate, it is necessary to use a large inoculum. It is evident from this work that large scale fermentation of the hydrolyzates is not without its difficulties and that further development of this step would be an aid to the practical success of the operation.

Heines and Nord (54a) described successive fermentation of Douglas fir hydrolyzate carried out with yeast and *Fusarium* mats to produce alcohol from hexoses and pentoses. Yeast fermentation of the hydrolyzates gave 89% alcohol of the yield theoretically expected from the hexoses and the subsequent *Fusarium* fermentation of the pentose residue gave yields up to 19% of those attained by the yeast.

**Engineering Aspects.** Studies of the chemical and engineering problems connected with wood hydrolysis are reported on by Plow, Saeman, Turner, and Sherrard (102) and by Saeman (110). The first of these papers deals with equipment and conditions of hydrolysis as it would be carried out in a plant, and the second deals with the kinetics of the hydrolysis process. In the latter paper, Saeman gives data on the hydrolysis of other cellulosic materials than wood, and considers the rate of breakdown of the formed sugar, since this is a very important factor in the economics of the process. The study of equipment was made on a spherical rotating digester. Digestions were carried out in a single step and by a multistage process. The effect of temperature, time of hydrolysis, acid concentration, liquid-solids ratio, and other process variables were investigated. Yields of 50-60 gal. of alcohol per ton of dry wood were obtained from multistage hydrolysis. However, 30 gal. per ton could be obtained from single stage hydrolysis which required only one-tenth the time necessary for the more complete hydrolysis.

*Economic Consideration of Production of Alcohol from Digested Wood.* Hasche (53) treats the fermentation of wood from an economic point of view in an excellent summary which emphasizes the relation of the utilization of by-products to the peacetime survival of hydrolysis processes. He

emphasizes the fact that the availability of "waste" wood does not mean wood at no cost. Assuming ethanol, tannin extracts, acetic acid, and furfural as products of a steaming, leaching, and saccharification process, Hasche concludes that it might be possible to carry out a moderate-sized operation on hardwoods, without sales credit for lignin. The profitable utilization of this wood constituent is the principal difficulty in devising an economic process for the use of wood. If the problem of utilizing lignin were solved and if it were thus possible to get a reasonable return from this product, which constitutes about 50–60% of the weight of the wood processed, the picture might well become favorable for wood processing by some of the methods developed in these wartime experiments.

#### *4. Sulfité Process Developments during the War*

The sulfité liquor fermentation process was carried out in two plants during the war period. One of these was the Ontario Paper Company's plant at Thorold, Ontario, Canada, and the other the plant operated at Bellingham, Washington, by the Puget Sound Pulp and Timber Company. The first of these is described by Callahan (26). In this plant, whose capacity is around 2200 gal. of 190-proof alcohol per day, the waste liquor is aerated to free it of excess sulfur dioxide and then neutralized with lime to a point suitable for yeast fermentation. Lime sludge is removed and the treated liquor pumped to wooden fermenters. The fermentation is said to be complete in about twenty hours, giving an alcohol concentration of 1%. From the fermenters, the beer is pumped to yeast separators and the yeast is separated for reuse in the fermentation. Beer freed of yeast passes to the stills where the alcohol is recovered. The probable cost, without charge for amortization, is given as 19.75 cents per gallon in a small installation and 12.04 cents per gallon in an average sized plant. At least 4 cents per gallon would have to be added to these figures to cover amortization. This plant is said to have operated well both from the fermentation and mechanical points of view.

At Bellingham, Washington, the Puget Sound Pulp and Timber Company operates a plant of about 5500 gal. daily capacity of 190-proof alcohol. This plant uses a process similar in some respects to the Thorold process. Liquor from the blow pits is freed of sulfur dioxide, cooled, and limed to adjust the acidity. Cooling at Bellingham is carried out in part by flashing the hot liquor *in vacuo*. This has the advantage of concentrating the fermentation solution somewhat, which is very desirable in a mash of such low sugar content (about 2%). The fermentation is carried out continu-

ously in wooden fermenters equipped with agitators to keep the yeast in suspension and thus permit the fermentation to go to completion. The fermenting solution flows through the battery of fermenters by a simple system of overflow lines from tank to tank and leaves the last fermenter to go to a small storage vessel from which the fermented sulfite liquor is distributed to yeast separators. The separated yeast, about one-tenth the fermented mash volume, is returned to the first fermenter in the series for reuse and the beer freed of yeast goes to the stills. The Bellingham plant is a very well designed installation and has operated quite satisfactorily.

**Technical and Economic Factors of Importance in Sulfite Fermentation.** It is interesting to consider the various factors, both technical and economic, that are important in sulfite fermentation. They can be set down with some degree of assurance on the basis of actual industrial operations such as the two just described. Considering first the technical aspects, the fermentation of sulfite liquor furnishes a good example of acclimatization of an organism to an unfavorable environment and of a means of maintaining this acclimatization under the day-to-day operating conditions in the plant. It was stated that both the plants described above reused the yeast. When a sulfite plant starts operating, the fermentation results are at first indifferent, but, by continued yeast separation and reuse, a body of yeast is built up that is able to function well in the liquor. This acclimatization to the liquor increases the alcohol produced by about 25% and, once attained, the higher production continues. The use of centrifugal separators is satisfactory mechanically for the job in hand. We thus have a good solution, both biological and mechanical, to the problem of acclimatization on a plant scale in the sulfite fermentation as carried out at Thorold and Bellingham.

Another interesting aspect of this fermentation is the continuous operation used at Bellingham. Sulfite liquor as delivered to the fermenters contains some sulfur dioxide, methanol, furfural, and various other substances which, taken together, impart considerable toxicity to the solution to be fermented. Reuse of the yeast gives an acclimatized organism for fermenting this toxic mash. In such a solution, however, other organisms which, if the mash were non-toxic, might constitute a serious infection hazard are unable to establish themselves. It thus becomes possible to run the fermenting mash continuously through a series of fermentation vessels without taking elaborate precaution to maintain the yeast as a pure culture. The sulfite fermentation when carried out in this manner shows the property of biological stability similar to that shown for instance by

such a fermentation as the one found in a vinegar generator. This state of affairs contrasts strongly with butyl alcohol fermentation which lacks stability as has been noted and where the maintenance of a pure culture calls for extreme precautions against infection. In such a case neither the metabolic products of the organism involved nor the nature of the fermentation solution provide any protection against infecting organisms.

As against the above-noted favorable factors in the fermentation itself, we have to place a number of unfavorable characteristics affecting the economics of sulfite liquor utilization that have to be given their proper weight in evaluating it. One of these is the low sugar content of the liquor from the pulp process. This runs from 2-2.5%, and the alcohol produced is, therefore, around 1% in concentration in the fermented mash. Recovery by distillation from this dilute mash requires about four times the steam used in the recovery of alcohol from the 7-9% beers of a grain or molasses operation. By the use of suitable heat economizers, this steam use can be about halved, but such an installation is more expensive. Also, the fermented mash in a sulfite liquor plant is corrosive and much of the equipment must be of metal resistant to a hot acid liquid containing free sulfur dioxide. The stills, economizers, and other equipment coming in contact with the fermented liquors should be made of stainless steel. As noted previously, the sulfite liquor contains traces of methanol which turns up in the stills and has to be rectified out. Bearing in mind the fact that ethyl alcohol may be used for pharmaceuticals, cosmetics, and food preparations, it is obvious that careful control of manufacturing operations is required to insure complete removal of methanol.

For maintenance of a sulfite fermentation operation of economic size, a correspondingly large pulp operation must be running in order to supply the liquor for the alcohol production. Furthermore, since the sulfite fermentation plant is inherently expensive on the distillation side, a considerable charge has to be made against the product for plant amortization costs. This can be reduced by spreading it over a number of years, provided the timbering operations can be counted on to last. Where there is a possibility that timber will be exhausted and the pulp operations moved elsewhere, a sulfite fermentation project would have to count on short-time amortization with consequent high product cost or on following the pulp mill to its new location. This in turn may separate the production widely from areas in which the alcohol is used, which adds to cost to the consumer by increase of freight charges.

It is such inescapable economic facts as those noted above, rather than

technical difficulties, that prevent wider utilization of the sulfite process. With high molasses and grain prices, however, the sulfite process may well give the cheapest fermentation alcohol. Also, if research on lignin develops uses for this material and its recovery from the alcohol plant stillage were feasible, the process would probably take its place permanently as a source of fermentation alcohol.

The fermentation of sulfite waste liquor was attacked in a novel way by Nord and co-workers and is reported by Nord and Mull (93). *Fusarium lini* Bolley ferments pentoses and was able to grow on sulfite liquor provided the liquor was first freed of toxic substances by lime treatment or, alternatively, by a resin. The amount of alcohol produced was small. Attempts were made to ferment with *Fusarium* stillage from an alcohol plant operating on wheat, and an amount of alcohol equivalent to 6–12% of that obtained from yeast fermentation was obtained. It was shown that *Fusarium* mycelium was not toxic to mice, which would indicate there was no objection to the use of a *Fusarium*-fermented stillage as feed.

### C. ALCOHOL FROM MOLASSES

Work on the technology of this process was very limited during the war period. The procedure is old and well worked out, and distilleries using molasses were more concerned with the question of obtaining the molasses than with refinements in the fermentation process. The equipment for ethyl alcohol production by fermentation of molasses is simple in design. Figure 4 shows a closed fermenter of 125,000-gal. working capacity in a molasses distillery. A few references of interest are noted in the paragraph immediately following.

Owen (99) studied the effect of bacterial contaminants on the efficiency of sugar utilization by yeast in molasses fermentations. His results did not yield a clear-cut indication that the contaminants were always harmful. Fluorides as antiseptics improved the efficiency in heavily contaminated mashes. However, they also had a beneficial effect in the same mashes sterilized by heat before seeding. The conclusion is that the effect of contaminants depends on the stage at which they are introduced and on the effect *per se* of the countermeasures taken. It must be borne in mind that other factors than the mere killing of the bacteria enter the picture. These other factors may themselves alter the course of the fermentation and thus make interpretation difficult.

Sattler and Zerban (111,112) have reported on a study of the unfermentable reducing substances in molasses. This is of considerable interest

to the fermentation industry, since a more complete knowledge of the unfermentables in this important raw material may indicate how their amount can be decreased. The nonfermentable reducing material in molasses may be as high as 17% in blackstrap and as low as 5% in high-test molasses. It is thus obvious that reduction of the nonfermentable substances would be of great economic importance. The work of Sattler and

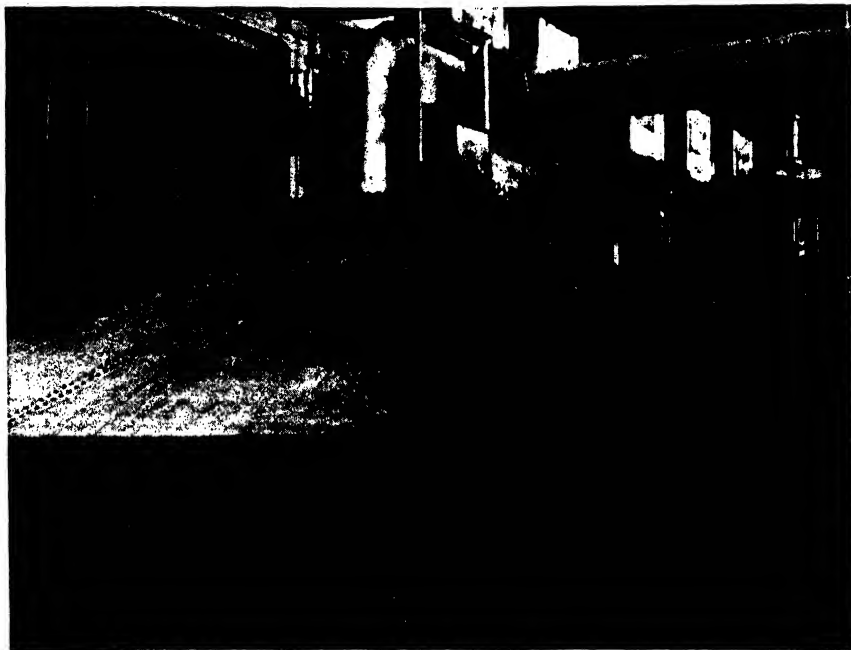


Fig. 4. Ethyl fermentation tank in molasses distillery  
(courtesy U. S. Industrial Chemicals, Inc.).

Zerban was carried out with this practical application in mind. They showed that the unfermentable substances were formed at the expense of the sugar, fructose especially being likely to undergo transformation to reducing compounds not available to yeast. If, for instance, a concentrated solution of fructose is boiled gently for a period of sixteen hours under reflux, a considerable fraction of the sugar undergoes dehydration and condensation reactions that yield nonfermentable reducing substances. Sattler and Zerban showed that the substance formerly called glucose consists

of a mixture of 1,2-fructopyranose anhydride, its nonreducing dimer, and dark-colored fructose caramel. Another type of reaction resulting in unfermentable substances is condensation between both glucose and fructose and the amino acids in cane juice. All of these reactions are accelerated by heat, and the authors call attention to the fact that both the high temperature used in processing cane juice in sugar mills and the subsequent storage of molasses under the hot tropical sun increase the amount of nonfermentable substances. Some injury due to heat is unavoidable in the process of sugar production; however, there is no question that the loss of fermentability in molasses could be decreased by cooling the final product before storage and by avoiding very high sugar concentrations in such products as high-test molasses. Sattler and Zerban found sugars could be regenerated from some of the condensation products by hydrolysis and suggest this might increase the alcohol yield in distilleries operating on molasses.

Hildebrandt and Erb (57) were granted a patent on a process for ethyl fermentation by yeast in which the yeast is grown in the final fermenter in a dilute aerated solution whose principal ingredient is stillage. This yeast-growing first stage is so managed that the entire quantity of yeast necessary for the final fermentation is produced before the sugar is added for alcohol production. After the yeast is grown, molasses or other sugar-bearing material is added rapidly, aeration is discontinued, and fermentation continues through a second alcohol-producing stage. This method of operation has been successfully employed where it is necessary to ferment solutions low in nutrients or in solutions not well suited to yeast growth. High-test molasses belongs to the first category and glycerol fermentations to the second. The production of glycerol by this process was patented by Hildebrandt and Erb (58).

An excellent compilation of references (124a) on yeast grew out of the wartime interest in yeast as a food. Although this use of yeast never grew to any proportions in this country, the bibliography referred to is invaluable to those interested in any aspect of yeast fermentation.

#### D. ALCOHOL FROM WHEAT

Prior to the emergency situation brought about by the war, wheat had not been used to any significant extent as a raw material for the production of industrial alcohol. Consequently, when the Federal Government decided to use surplus stocks of wheat in the alcohol program as a basic material *in lieu of corn*, it was necessary to devise methods of processing



this unfamiliar material to avoid losses, delays in production, and general plant difficulties.

A wheat-alcohol research committee was formed by the War Production Board and, through this committee, public-spirited groups of scientists and industrialists co-operated in solving problems and establishing adequate operating technics to meet the situation. The Northern Regional Research Laboratory acted as a co-ordinating agency in this undertaking and members of the laboratory staff made valuable contributions to the solution of problems arising in connection with the use of wheat. Three wheat-alcohol conferences were held at the laboratory in November, 1942, February and September, 1943. At these meetings scientific groups and industrial representatives attended, and there was free interchange of information by discussion as well as by means of formal reports submitted by those interested. These reports were made for the information of producers and at present constitute part of the records of the war activities on file with government agencies. Copies of the reports were widely distributed among the groups concerned, and some of them have been released for publication. Those released are available in the Department of Commerce, Office of Technical Services, Industrial Research and Development Division, Washington, D. C.

A review of fermentation developments during the war would be incomplete without some discussion of fermentation alcohol from wheat and the importance of alcohol as a raw material for war essentials. Chief among these uses was its conversion to butadiene for synthetic rubber. Whatever may be the future of this particular use of alcohol, it was of tremendous value during the war. The program of obtaining alcohol by fermentation of wheat thus becomes an essential part of the war picture and has both technical and historical interest. This production was possible in large part because beverage alcohol plants could be utilized and because the country was fortunate in having a surplus of grain usable in these plants.

### *1. Technology of Use of Wheat in Distilleries*

Unfamiliarity with wheat led many technologists to expect large losses as a consequence of using it in place of corn. When its use was first suggested, lowered yields, difficulty with stills, and more serious difficulty with by-product recovery processes were anticipated. These problems, which appeared formidable at the outset, were solved more promptly than was expected, and the distilling industry was soon producing large quantities of alcohol from this grain. As an indication of the magnitude of this production, attention may be called to the 107,000,000 bushels of wheat used from the 1943-44 crop. This amount produced about 250,000,000 gallons of 95% alcohol, which is almost twice the prewar peacetime production of industrial alcohol from all raw materials<sub>2</sub>(64a).

Some general comments on the technical points involved in the use of this grain in the principal distillery operations are: *Grinding*—usual grinds are satisfactory; very fine grinding is to be avoided. *Cooking*—cooking should be carried out preferably above 20 gal. per bushel, with pH between 5.6 and 6.0 and temperatures around 300°F.; high-temperature cooking lessens foaming in stills and evaporators. *Converting*—if low-temperature mashing is used, antiseptics are necessary; 1% premalt and 6–7% converting malt are ample. *Yeasting*—yeast equivalent to 3% of mash volume is satisfactory. *Fermentation control*—pH should be held around 4.5 and should not go below 4.0 at the end; temperature should not exceed 89°F. *Distillation*—no different from corn except that wheat requires more frequent cleaning of beer stills than corn; this is due to fouling of plates, especially on the underside. *By-products recovery*—may be carried out in conventional equipment; owing to the glutinous nature of wheat, the recoveries are decreased when wheat is used and plant operations are more difficult.

Thorne *et al.* (129) reported a method for the evaluation of malts for the production of alcohol from wheat. The interesting feature of their paper is the conclusion that malt is best evaluated by an actual fermentation test. They support this by showing that there is only a limited correlation between the conventional Lintner values and actual alcohol production. While it is helpful to know the Lintner value, a precise evaluation of the malt can be made only from the fermentation itself. Details of a suitable test are given in the publication.

It is generally felt that the most desirable way to use wheat is in admixture with corn or milo. Mixtures of grains lessen the difficulties peculiar to wheat, and plant operation on such mixtures is not significantly different from that on corn alone.

## *2. Disposal of Spent Grains from Wheat Fermentation and Value as Compared with Corn*

Since disposal of spent grains is so important in the operation of a distillery, the use of wheat required investigation of feeding value of residues from the fermentation. Corn residues have had a place in mixed feed for many years and may be taken as a standard by which to evaluate other distillers' grains. O. P. R. D. therefore sponsored a study by the University of Wisconsin (contract W. P. B. #70) to demonstrate the comparative value of wheat and corn distillery residue grains.

Tests were made on Holstein cows at each of two stations, putting groups on both types of grain. The feed mixture contained 40% of the distillers' grain under test and was fed with mixed hay and silage to provide roughage. The two types of distillers' grains gave mixed feeds of equal palatability, and weight was either maintained on both types of feed, or a slight gain was shown. Milk production was slightly greater on corn distillers' grains than on wheat distillers' grains, the difference amounting to about 3%.

On the basis of these results, and considering the relative efficiencies of utilization of the feeds, it was concluded that wheat distillers' grains at \$38.00 per ton was about equivalent to corn distillers' grains at \$44.00 per ton.

### *3. Use of Granular Wheat Flour or Alcomeal*

A wartime development leading to the production of very large gallon-ages of alcohol from wheat was the use of "granular flour," sometimes called Alcomeal, in molasses distilleries. The war requirements found these plants entirely unequipped for the mashing of grain. Furthermore, the necessary equipment for grain mashing was not available and there was no time to fit up the plants for grain handling even if equipment could be found. In this emergency, it was found possible to use a coarse, partly milled granular flour by slurring it in warm water, heating to approximately 150°F. for malting, then cooling and fermenting. This process wasted large amounts of wheat protein in the discarded stillage but some of the feed values were saved by the preliminary milling. It was susceptible to contamination since the grain was not pressure-cooked and spore-forming organisms were carried into the fermentation. By using antiseptics such as fluorides and chlorinated phenols, it was possible to hold the contamination down to a degree which permitted a reasonably satisfactory operation. Approximately 10 to 10.5 proof gallons could be obtained per 100 lb. of dry flour. These developments were described by Singleton (122) and by Haywood, Emerson, and Davis (54). Large quantities of granular flour were also used by conventional pressure-cooking methods, either alone or in combination with other grains.

### *4. The Balls-Tucker Process*

As a possible means of utilizing wheat and at the same time conserving malt, Balls and Tucker (9) of the United States Department of Agriculture suggested using a dilute sulfite solution to extract the diastase from ground whole wheat or wheat flour. Enzyme so extracted may be used to digest the starch of the grain in place of a considerable portion of the malt which would otherwise be necessary. This suggestion was of interest not only because it economized malt, but because it appeared to make possible the recovery of gluten from granular wheat flour mashing since in sulfite-treated mashes the gluten floated to the top of the mashing vessel and could be skimmed off. The process was investigated by various groups interested in the fermentation of wheat and was used in large-scale operations in certain distilleries. Recoveries of gluten under the conditions

obtaining in wartime operation were not up to the original expectations and this feature was therefore not put to practical use.

The general method of mashing followed in the Balls-Tucker process was to put 25% of the total grain in an agitated vessel with warm water and enough sodium sulfite to give a 0.05 to 0.1% solution, add the malt necessary for the process (about one-fifth to one-third the usual amount), agitate, allow to settle, and decant the supernatant liquid. The settlings were combined with the remainder of the grain and sent to the cooker. After cooling the cooked mash, the decanted solution was recombined with it and digestion of the starch was allowed to take place by the combined malt and wheat enzymes. By thus supplementing the extracted wheat enzymes with malt, satisfactory alcohol yields were obtained. At least 25% of the wheat used in the mash had to be extracted in order to get sufficient diastase to convert the remainder of the mash. Balls and co-workers have reported further (8) on this process.

#### E. DEVELOPMENT OF MOLD BRAN AND RELATED PROCESSES

The war stimulated considerably the development and use of enzymic preparations other than malt for saccharification of starchy grains. The most likely method of producing such enzyme preparations is the use of amylolytic molds such as those employed in the Orient for the production of sake (rice wine). This modern application of a very ancient art was brought about during the war period by a shortage of malt consequent on the great increase of fermentation alcohol from grain. Had the making of beverage alcohol not been sharply curtailed during the war, the capacity for malt manufacture would not have been adequate to fill these requirements.

In the United States, credit for introducing amylases made from molds must be given to Takamine who came to this country in 1891 with the intention of making a malt substitute for use in brewing and in the distilled beverage industry. While he was not able to do this, he did set up a process which has been successful industrially for the manufacture of diastase produced by the growth of mold. Through publication (126) and patents (127) he outlined the essentials of manufacture of mold amylase made by growing the fungus on bran or other suitable substrate, giving details of the methods of growth and also of the procedure by which the enzymes were extracted and put in dry form. The methods of preparing mold amylase products during recent years has been for the most part along the lines outlined by Takamine with only minor modifications.

The use of a product made by growing a suitable mold on bran, drying the preparation at the height of its amylolytic activity, and using this material in a manner similar to dry malt has been felt to be the most practicable way to utilize the starch-digesting power of molds in fermentation technology. This feeling is no doubt based on the fact that use of such a preparation falls in with accepted practices and thus makes for a minimum of change in the orthodox fermentation plant procedures. Noteworthy

progress has been made by investigators in recent years in the testing of various molds and mold preparations in grain mash.

The most active centers for work on the use of mold as a converting agent have been in the grain-producing regions of the middle west. University and government laboratories and research laboratories of the distilling industry contributed to the development. In 1939, Underkoffler, Fulmer, and Schoene (133) published a report on the use of preparations made from *Aspergillus oryzae* for the saccharification of corn mash. Alcohol yield was excellent with use of this preparation which consisted of a wheat bran on which the fungus had been grown.

The bran was first sterilized in drums, then cooled and inoculated with mold spores. The drum containing inoculated bran was rotated while introducing sterile air to promote mycelial growth. After about forty hours, rotation was stopped and the bran was dried at room temperature by spreading it in layers on paper. This dry product used in amounts equivalent to 5-10% of the corn in the mash gave good yields of alcohol.

The above work was extended by the same group of workers in 1940 (117), and, with a view to developing the commercial possibilities, the moldy bran was used on an acid-hydrolyzed mash of ground corn. Such mashes do not give a good alcohol yield in spite of the apparently nearly complete conversion of starch to sugar. With moldy bran added, however, the alcohol yield came up to the best obtained by the use of malt. Combinations of the mold preparations with malt and soybean meal were also tried, with yields usually higher from the combinations than from the materials used singly. The improvement with mixtures was interpreted as due to increase in the number of enzymes present.

Hao, Fulmer, and Underkoffler (49) studied the use of mold bran produced from a large number of amylolytic fungi by an improved laboratory technic. Batches from 27 strains of molds of four different genera were used to saccharify mashes made from pressure-cooked ground yellow corn. Low temperature saccharification (30°C.) proved satisfactory. *Aspergillus oryzae* was the best of the molds tried from the point of view of yield and ease of handling. In this investigation no malt was used and rather high percentages of mold bran were required to obtain the maximum alcohol yields of 95% of theory. It was found that there was no deterioration of the product on storage if the moisture content did not exceed 12%. The amylase activity increased by the addition of iron and zinc salts to the bran made from *Aspergillus* species but the salts were disadvantageous in the case of *Rhizopus* species.

This study of the use of mold bran and acid hydrolysis was carried further by Goering (46). He worked on corn, corn starch, and cassava starch, first hydrolyzing with acids and then fermenting. Mold bran was used in addition to hydrolysis in some experiments. Goering observed that hydrolysis of corn meal gave a toxic material due to acid action on the corn bran. Mold bran increased the yield of alcohol from the acid-hydrolyzed starch mashers but malt did not, thus adding evidence to support the superior converting action of mold amylase. Underkofler, Goering, and Buckaloo (134) investigated the effectiveness of various mold amylase preparations, finding them superior to malt. The Lintner value, which is useful in the case of malt as an index of saccharifying power, was meaningless for the mold preparations. It was concluded that fermentation tests are the only adequate measure of the activity of mold amylase preparations. In 1942 Underkofler (131) gave a general summary with some 25 references of the status of the mold amylase development at that time.

The question naturally arises as to relative effectiveness of various molds known to have amylolytic power. Underkofler and Fulmer (132) studied this and found no marked difference among *Penicillium*, *Aspergillus*, *Rhizopus*, and *Mucor* species. A significant increase in amylolytic power was produced by trace elements in the case of certain molds. The synergistic effect of a mixture of enzymes is noted by Oesting (95), who recommends the inclusion of proteases in the mixture. Hao and Jump (10) undertook a study of the mold and bacterial amylase preparations available on the market or potentially available. Their object was to settle the relative merit of these products as compared to malt. The authors covered several important aspects of the use of these substances. First, they showed the effect of using the various converting agents simply as liquefying materials, then, of finishing the conversion with a single agent, "Converzyme." Successful liquefaction was obtained in all cases. The various agents were then tested for converting power and were shown to be equivalent to malt. Combinations of mold and malt were used with the same result as reported elsewhere—that combinations gave yields above those obtained when preparations were used singly. It was concluded that the various preparations were satisfactory converting agents but in most cases their cost put them out of the economic range for use in grain distilleries.

An interesting contribution to this subject was made by Beckord, Kneen, and Lewis (12). These workers produced an amylolytic product by growing *Bacillus subtilis* on wheat bran moistened with a buffer solution.

This medium was chosen so that the bran could be dried and preserved for use after growth of the organism to give a product similar to malt. They worked out the optimum conditions for growth and tested action on starch by measures of dextrinization and used yeast fermentation as an index of sugar production. Under the best conditions their product was slightly better than barley malt by both criteria.

Beckord, Peltier, and Kneen (13) extended the work on *B. subtilis* by producing the amylase on thin stillage. In order to obtain a significant amount of enzyme, rather definite conditions had to be established. The organism used forms a pellicle which should be maintained intact for best results. The enzyme had to be kept distributed through the medium, and fresh nutrient had to be brought to the surface of the pellicle. This was accomplished by mild agitation and continuous flow of the nutrient solution. An apparatus similar to a quick vinegar generator was also tried. An important feature of this paper is that the authors attempted to use thin stillage for the production of their converting agent. If this could be done, an alcohol process operating on starchy materials might be made self-sufficient so far as the operation of converting the starch to sugar is concerned.

Under the sponsorship of O. P. R. D. (contract W. P. B. #54), the University of Nebraska undertook to develop a plant set-up for mold bran production. This project was carried out by L. M. Christensen, Kenneth J. Goering, G. M. Severson, and L. A. Underkofler. Experimentation was carried to the point at which a definite flow sheet for a small producing plant could be laid out.

The plant included a continuous rotary cooker followed by a cooler for the preparation of the bran for the inoculum. After inoculation, the mold was allowed to grow for a time in stationary incubation cells. Later it was transferred to a secondary rotating incubator, from which it was introduced in the moist state directly into the alcohol mash, thus avoiding the necessity to dry the product. The problem of keeping the mass of inoculated bran from overheating during growth is a formidable one and was solved by using large quantities of cool, humidified air. In the plant sponsored by the government for wartime alcohol production from grain at Omaha, Nebraska, a mold bran installation somewhat simpler than the one noted above was in operation. This plant included a dryer and the dried product was produced in quantities sufficient for large scale tests. These tests substantiated in a general way the findings from laboratory runs.

The general economic and operating aspects of the subject were covered by Boyer and Underkofler (21). They conclude that costs of malt and mold bran are comparable and that there is a gain in fermentation time and an increase in yield when the bran is used. Christensen (27) has

taken out a number of patents on process and apparatus details connected with mold bran production.

In 1909, a patent was granted to Woolner and Lassloffy (149) on a process for the manufacture of a converting agent for starchy mashes using thin stillage as a substrate for growth of the amylase-producing agent, in this case *Aspergillus oryzae*. The patentees stated that it is necessary to keep the stillage protected against bacterial infection. They proposed to replace part of the malt with the mold stillage which, judging by the quantities given in the patent, would not appear particularly active as a saccharifying agent. They call attention to the advantages of mold and malt together over either one used alone. This process was considered by the workers at the University of Nebraska, but was not believed practical. They also considered the amylo process and rejected it as lacking practicality.

Recently, Erb and Hildbrandt (39) reported operation of a distillery on mold grown in stillage. The plant in question was running on granular wheat flour and the period of testing was, unfortunately, short but the results were good. These authors found that the growth of mold in stillage was markedly stimulated by traces of metallic aluminum. They were able to produce large amounts of mycelium which under proper conditions was quite active and capable of replacing most of the malt. The mold growth stage in the stillage was carried out as a pure culture but the yeast fermentation was under the same conditions as those used when malt was employed along, namely, without pure culture precautions.

The use of mold as a malt adjunct is one of the promising new developments of the war period. Until industrial alcohol rather than potable spirits was the product desired from grain fermentation, distillers had little interest in processes using mold because of the change in flavor of beverage produced by converting starchy mashes with mold rather than malt. In view of the great advances in knowledge of deep vat mold fermentations and the widespread use of this method for the growth of mold mycelium, it should be possible for distillers to grow amylolytic molds for use in conversion. When we add to this possibility the fact that these molds will grow on stillage, there is a good prospect of making industrial alcohol from starchy mashes without the use of any converting agent other than the mold produced by the distillery. As is noted in many of the references above, a small amount of malt might be desirable even if not absolutely necessary since the various enzymes have a synergistic effect and maximum yields are obtained only from combinations of converting agents.



## F. GLYCEROL FERMENTATION

Owing to its use in explosives, glycerol was required in large amounts during the war. In the beginning of the conflict especially, the shortage was critical, and in both Britain and the United States the possibility of manufacture by fermentation was considered. During the first world war, large quantities of glycerol were produced by fermentation in Germany, using sugar as a raw material and sulfites as steering agents; however, early in the present war, sugar for human food became scarce, thus limiting its use for fermentation purposes and making it unavailable for glycerol manufacture except in case of great necessity. This meant that whatever raw materials were available for alcohol production would have to serve also for the fermentation production of glycerol.

If glycerol has to be produced by the fermentation of molasses or grains, the problem of recovery becomes very formidable, so formidable that lack of a workable and economical recovery process has so far prevented the use of either of these as raw materials in competition with glycerol from fats. Not only is recovery difficult, but, in the case of grain, a preliminary acid hydrolysis of the starch would be necessary to produce sugar for the fermentation. Because of this, grain has not hitherto been considered as a possibility for glycerol production. During the war, however, wheat was to be had in large quantities, and a process for glycerol from this grain was suggested. If molasses were used considerable residue would be produced and this, unless salable, would constitute a heavy disposal charge on the process. Many fermentation glycerol processes call for large sulfite or alkali additions; residues from such processes would be unfit for fertilizer which is the most likely outlet. Considering all the circumstances molasses seemed to be the raw material which would probably be used if at all possible.

Recovery of glycerol from molasses stillage requires first, evaporation to bring the total glycerol and solids to a concentration of about 50% by weight. This requires only standard evaporating equipment and no special problems are involved. Depending on fermentation methods and type of molasses used, the nonaqueous 50% of the concentrated stillage may contain from 20–40% of glycerol. Since glycerol boils at high temperature, separation by ordinary distillation is not practical. The water boils off first and leaves a tarry mass which cannot be handled in any known type of still. Extraction may be used, but it is difficult to find a solvent sufficiently selective to take out glycerol and leave behind the numerous water-soluble substances derived from the molasses. Glycerol

is similar in its solubility relationships to water, and solvents not miscible with water have no affinity for glycerol, while those miscible with water dissolve glycerol but also dissolve many of the associated water-soluble impurities. Thus, a process for production of molasses fermentation glycerol has to fulfil the following requirements: (1) The fermentation process should be such that the final residue can be disposed of as a fertilizer thus reclaiming the potash value of the molasses, and, what is equally important, relieving the process of an actual disposal charge. (2) A practical method must be available for making the first separation of glycerol from the solids of the concentrated stillage. (3) The first separation procedure must deliver a crude glycerol capable of being refined at reasonable cost.

These requirements are well known to those who have attempted to develop the industrial production of fermentation glycerol. They were not significantly altered by the fact that, during the war, the economic aspect took on minor importance, since they are concerned primarily with the technology of the fermentation and subsequent glycerol recovery. They constitute interesting guideposts in reviewing the wartime developments in this field.

**Glycerol Fermentation Studies.** The fermentation developments reported in recent years rely for the most part on well-known procedures for increasing the amount of glycerol produced by yeast. Cane juice, cane molasses, and beet molasses are among the raw materials proposed for use. Iwata (64) describes a process for cane juice treated with considerable quantities of sodium sulfite. Glycerol is extracted with a mixture of absolute alcohol and carbon tetrachloride. Owen (98) and Owen *et al.* (100) discuss the fermentation with sulfites and alkalis as steering agents. It is stated that this method of producing glycerol has good possibilities, but nothing significant is added to recovery methods by these authors. A German-patented process (94) suggests the use of magnesium carbonate as a neutralizer for acids formed during the fermentation. Rao (103) reports on the fermentation of molasses made alkaline with carbonates or alkaline sulfites. Glycerol is recovered by extraction. Mnookin (88) has patented a process which involves fermenting blackstrap molasses containing sulfite and vacuum distilling to remove the alcohol; then fresh fermentable material is added and fermentation is continued. By this means it is proposed to build up the concentration of glycerol to a point where recovery would be possible. Duchenne (37) describes a process for obtaining glycerol by fermentation of a 21° Brix syrup and implies the process can be used for molasses. Fermentation of a concentrated solution of sugar and

nutrients in the presence of small amounts of sulfur, sulfides, or sulfur-containing organic compounds with constant aeration is claimed by a German patent (63) to give commercial amounts of glycerol. Neish, Blackwood, and Ledingham (92) describe a fermentation by a *B. subtilis* strain producing glycerol and 2,3-butylene glycol as main products from a glucose solution. Lees (74) describes a process involving sulfites as steering agents and shows the relationship of amount of sulfite and amount of yeast inoculum to glycerol production in pure sugar solutions. Fulmer, Underkoffler, and Hickey (44) took out a patent on a fermentation process using pure sugar and various sparingly soluble sulfites as steering agents. They claim fermentation on the acid side.

Hodge (61) describes a molasses process in which the yeast is grown with aeration in a preliminary dilute stage, in the main fermentation vessel. The larger portion of the molasses is then added and the fermentation is carried out at a pH of about 6.8, regulation of the acidity being accomplished by the addition of lime and ammonia. This procedure gives about three grams of glycerol and six grams of alcohol per hundred cubic centimeters from a solution containing about eighteen grams of sugar per hundred cubic centimeters. It is to be noted that the residue from this fermentation would contain no toxic materials and therefore could be dried and used as a fertilizer. The procedure provides for disposal of waste and also furnishes a good medium for the yeast, both of which are extremely important for success in an industrial operation.

**Recovery of Glycerol from Fermentation Solutions.** The recovery of glycerol from fermentation solutions containing pure sugar, yeast nutrients, and the required steering agents is not unduly troublesome. However, when commercially usable raw materials are employed, recovery poses a difficult problem as is proved by numerous patents on recovery processes. Separation of a high-boiling substance like glycerol from a water solution containing mineral salts and other non-volatile solids by distillation runs into mechanical difficulties that have to be attacked by special devices.

One method of distilling is breaking the glycerol-containing concentrate into a fine spray under conditions such that the glycerol and water are flash distilled, leaving the remainder of the solids in a dehydrated form for recovery. Two methods of carrying out this type of processing have been patented in recent years. One, described by Dennis (33), uses a spray-drying apparatus modified to run at a very high temperature (1100-1200°F.) with an oxygen-free furnace gas as the heat transfer medium. Glycerol-containing concentrate is sprayed into the inert gas stream at such a rate that evaporation reduces the temperature of the exit gas to 400°F. The dry solids are separated

from the gas stream in dust separators and the glycerol then is washed out of the gas in suitable apparatus. The recovered solids are valuable as a fertilizer. A second method is covered in two patents issued to Hildebrandt (55,56). This method proposes to introduce the concentrated glycerol-containing fermentation residue in a continuous stream into a body of heavy oil heated to about 400°F. Instantaneous flash evaporation of water and glycerol takes place, and the nonvolatile solids remain in the oil from which they can be separated for fertilizer use, while the glycerol, water, and other volatiles pass out of the oil bath and may be condensed. Oil distilled over is decanted and put back into the system and glycerol is evaporated to concentrate it for subsequent purification. Both the Dennis and the Hildebrandt processes operate at atmospheric pressure. Neither of them has reached the stage of industrial application.

Another line of attack on this problem is illustrated by the work of Walmsley (138) who evaporates the fermentation residue, then adds lime in large amount so that the mixture becomes semisolid. This mixture is extracted with alcohol and the extract is heated to evaporate the solvent and give a crude glycerol. The crude product is then extracted with aniline. From the aniline, the glycerol is washed out with water and purified. A similar process has been patented by Walmsley and Davis (139) for the recovery of 2,3-butylene glycol. Batchelder and Peterson (11) propose to recover the glycerol by liquid-liquid extraction of the concentrated residue with acetone. The difficulty here is to make the solvent sufficiently selective to dissolve the glycerol without dissolving significant amounts of impurities. This the authors propose to do by careful regulation of the ratio of acetone to water and solids. Under proper conditions, a two-layered separation can be made with the upper layer containing all the glycerol and only about one-fourth of the solids of the original fermentation concentrate. This process does not make the complete separation necessary for glycerol recovery, but is doubtless a part of a more comprehensive recovery scheme.

Cornwell (29) has patented a process for production of a plasticizer for cellophane which consists in making a glycerol fermentation in a sugar solution kept alkaline by potassium carbonate additions. The potassium acetate formed is hygroscopic and serves along with the glycerol to plasticize the film. It is proposed to remove the glycerol and the potassium salt from the fermentation solution by dialysis. Cornwell (30) also secured a patent on the recovery of 2,3-butylene glycol by dialysis and subsequent purification by base exchange compounds. Wallerstein, Farber, and Dayton (137) secured a patent on a process designed to utilize grains for glycerol production. Their procedure is to digest the starch first, enzymically, then to use a solution of ligninsulfonic acid to precipitate protein

from the digested solution. Hydrolysis of the starch is then completed with acid, and a glycerol fermentation is carried out in the presence of steering agents. Glycerol is recovered by distilling the concentrated residues with superheated steam in a vacuum.

Industrial production of glycerol from fermentation has not yet become an actuality in the United States, but some of the processes described above have merit. With the coming of more normal economic conditions and when lower cost sugar becomes available in the form of molasses, or otherwise, fermentation glycerol might become a commercial possibility.

#### G. DEVELOPMENTS TOWARD USE OF CONTINUOUS FERMENTATION METHODS

The fermentation process as ordinarily carried out is a batch process, although considerable attention has been given in the past to setting up fermentation on a continuous basis. During the war, contributions from the research department of Joseph E. Seagram & Sons, Inc., added to the information available on this subject. Especially valuable was the working out of a method of cooking grain based on the introduction of high pressure steam and mash together into a mixing jet which discharged into a relatively small holding tank. This equipment replaced the bulky cookers with motor-driven stirrers used in the grain process run according to orthodox methods. On account of the short retention time in the system (less than three minutes at about 360°F.) large cooking capacity could be set up with a minimum of equipment. The use of this high temperature "flash" cooking greatly facilitated the construction of wartime grain alcohol production plants, because it made possible the processing of very large quantities of grain without requiring steel for tanks and large motors for stirrers. Both steel and electric motors were in short supply when the alcohol facilities were in need of expansion. Mash cooked in this fashion may be cooled by first flashing to a low pressure in a tank following the cooker, then by subjecting the partly cooled mash to vacuum evaporation to bring it to malting temperature. Malting is carried out continuously for a short period and the mash is cooled and discharged to fermenters. It is readily fermented to good yields of alcohol. This system is described by Willkie and Kolachov (147) and by Unger, Willkie, and Blankmeyer (136). The application of the continuous cooker to wheat is described by Stark, Kolachov, and Willkie (124).

The Seagram group published on various aspects of the continuous fermentation scheme. With the cooking step made continuous, the question arises whether the other steps in the mashing and fermentation can

likewise be carried out in this manner. The malting step is taken up by Gallagher, Bilford, Stark, and Kolachov (45). By pumping slurried malt continuously into the stream of mash from the cooker after cooling the mash to the malting temperature, satisfactory conversion was obtained in a few minutes. The malted mash was then cooled and put into the fermenter. Unger, Stark, Scalf, and Kolachov (135) published a tentative flow sheet for continuous yeast production from grain. Very little operation data were given, and the authors state that the system would have to be tried for an extended period to prove its worth. Molasses fermentations were conducted continuously and reported on by Bilford, Scalf, Stark, and Kolachov (14). These were laboratory fermentations made in a continuous manner in a wide-mouthed Pyrex jar. Analyses on samples taken at intervals determined the rate at which fresh mash could be fed and fermented mash drawn off. Yeast cell counts were made and relation of cell count to fermentation rate was established. It was suggested that large scale fermentations could be carried out by this system. The "flash" conversion by malt and certain other features are covered in a patent issued to Blankmeyer and Stark (17).

Continuous manufacturing processes are the order of the day. Where sufficient advantage is gained by their use, they will undoubtedly become established. It will be of interest to see whether the procedures noted above can be carried into actual plant operation since only an extended period of plant use would show whether they have any hidden weaknesses. As has been pointed out in the case of the sulfite process, there are conditions under which continuous operation results in improved performance. However, where acclimatization is not important and where a very highly nutrient medium is involved as is the case in the grain fermentation, it is possible that contamination might enter the system and ultimately build up to the point where the continuous operation would become impractical. It is true that a continuous plant would be small and compact for the capacity involved but many mechanical complications are introduced by the necessity for maintaining the system infection-free. It is to be hoped that this group of workers will report the results of actual large-scale trial of these interesting suggestions.

#### IV. 2,3-Butylene Glycol

##### A. RELATION TO WAR EFFORT

Among the wartime fermentation developments was a renewal of interest in 2,3-butyleneglycol fermentation. This substance can be used as a

raw material for the production of butadiene, the need for which early in the war as a synthetic rubber raw material led to the exploration of every avenue by which it might be produced. In the early stages of the rubber development, certain difficulties arose due to impurities in the butadiene available, and it was felt that the product made from fermentation glycol would be of high purity and therefore better adapted to polymerization than the butadiene from other sources. Tests made on butadiene from fermentation glycol subsequently verified this fact.

Government interest in developing all possible aids to rubber production led to a meeting of representatives of the fermentation industry at the Northern Regional Research Laboratory in April, 1942, to discuss the possibility of producing 2,3-butylene glycol by fermentation. This was followed by meetings in June, 1942, and August, 1942. The interest stimulated by these meetings plus the drive of war needs resulted in a co-operative and systematic attack by a number of government and private agencies on the problems of the fermentation and recovery of glycol from the fermentation liquor and the production of butadiene from the glycol so produced. This work is felt to be of sufficient interest to warrant a review in some detail, since it illustrates the type of investigation which may be necessary to launch an industrial fermentative process.

Like many other bacterial processes of industrial interest, the 2,3-butylene glycol fermentation had been studied bacteriologically and many of the difficulties of the fermentation had been overcome, but no systematic investigation had been made of recovery methods for the glycol produced. Without these studies production of glycol industrially was not possible, since the desired product is high boiling, and is not removable by ordinary methods of distillation. The efforts of groups interested in this general problem answered many of the questions as to removal and subsequent processing and provided a basis for the complete series of production steps from glycol fermentation to butadiene production as a stand-by process for butadiene. The work was, therefore, successful, although it was admittedly not economically attractive. In the treatment following, the fermentation of sugar and starch are first considered, then the work on recovery, and finally the chemical steps necessary to produce butadiene from the glycol. All of these were investigated on the pilot plant scale by one or another of the interested groups involved in the general project.

## B. FERMENTATION INVESTIGATIONS

When war work on the butylene glycol production was started, two bacterial fermentations were known that yielded substantial amounts of this substance. In 1906, Harden and Walpole (51) had reported on the *Aerobacter aerogenes* fermentation. Scheffer (113) extended this study in

1928, and in 1933 Fulmer, Christensen, and Kendall (43) at Iowa State College showed that yields were possible from the *aerogenes* fermentation which gave promise of industrial application. This organism yields mainly a mixture of the optically active dextro and the meso forms, with a preponderance of the latter, together with some ethyl alcohol and acetylmethylcarbinol. Since the organism has no diastatic powers, this fermentation can only be run on sugars. The other 2,3-butylene glycol fermentation carried out by *Bacillus polymyxa* had been investigated by Donker (36) in 1926. This organism is actively diastatic and thus can ferment starch. It has been found to produce the *l*-isomer exclusively. It also produces ethyl alcohol. The two known fermentations thus furnished methods for the production of glycol from either sugar or starch provided they could be proved practicable. Early in the war a surplus of wheat in the United States and Canada lent emphasis to the desirability of a direct starch fermentation, but the sugar organism was better known and, as a matter of fact, proved subsequently to be better adapted to industrial use. One of the first studies undertaken following the decision to capitalize on the possibilities of 2,3-butylene glycol as a source of butadiene had to do with the products of the two organisms and their physical characteristics. These studies were made by Ward and co-workers (142), who investigated the optical isomers produced by the 2,3-butylene glycol fermentation. From sugary substrates *A. aerogenes* produces the meso-dextro mixture. This mixture has a tendency to crystallize at room temperature, is high in viscosity (118 centipoises at 25°C.) and forms a pentahydrate. *B. polymyxa* acts directly on starchy mashes and, in contrast to *A. aerogenes*, produces levo 2,3-butylene glycol. This glycol is significantly different in physical properties from the meso-dextro mixture. It has a viscosity of 41 centipoises at 25°C., remains liquid at low temperatures, and does not form a hydrate. The fact that the glycol remains liquid at low temperature suggests the possibility of using it as an antifreeze.

### 1. Industrial Development of Sugar Fermentation by *Aerobacter aerogenes*

A brief résumé of the industrial progress on 2,3-butylene glycol fermentation might begin with comments on U. S. patents issued to Kluyver and Scheffer of Holland. The first of these patents (69) covers the fermentation of a carbohydrate by *A. aerogenes* using very large quantities of air. It is directed toward the production of acetylmethylcarbinol rather than 2,3-butylene glycol which is produced with lower aeration rates. In the process described in a second patent (70) the same organism, *A. aerogenes*,



is used to produce 2,3-butylene glycol. The growing solution is made up essentially of a fermentable carbohydrate, a nitrogenous nutrient, a phosphate, and a carbonate for neutralization. This patent covers a wide field since it deals with both starches and sugars and also mentions the use of the starch-fermenting *B. polymyxa*. It contains eighteen claims, only a few of which are supported by the two examples given in the specifications. In a third patent issued to Scheffer (114) it is stated that the principal importance of aeration in this process is to rid the fermentation of the carbon dioxide evolved. He claims a process in which the fermentations are agitated with an inert gas instead of air.

Until war requirements made the necessity of large-scale manufacture of butadiene from glycol a possibility, there was no active industrial interest in the United States in either fermentation process. Some small-scale experimental work had been done in the research department of one of the chemical companies (55) in 1940-41 on the fermentation of molasses by *A. aerogenes*. This was carried out to produce enough glycol for laboratory experiments on the production of butadiene and to see whether a pilot plant operation could be run on the process as patented. It was found that the process described in the patent had to be modified to get satisfactory pilot plant results. In contrast to the patent claims, the pilot runs showed that the nutrients could be greatly reduced in amount, that the organisms could not ferment sugar concentrations above 10%, that aeration rates could be substantially reduced under proper conditions, and that the yields were not as high as claimed. Typical runs gave total products equivalent to 40% of the sugar of which 28% was glycol and 12% ethyl alcohol.

Torres and Frias (130) describe a fermentation of molasses (13-14° Brix) for the production of 2,3-butylene glycol. The molasses is adjusted to pH 6.0-6.2 and fermented for 36 hours at 31-32°C. The mash is aerated at the rate of six cubic feet per thousand gallons per hour. When sugar content is decreasing at the rate of 0.1% per hour, aeration is discontinued. The pH decreases slowly during the fermentation. The authors state that the sugar should decrease gradually, or there will be an unduly high production of acetylmethylcarbinol. They also call attention to the fact that mechanical agitation permits a lowered aeration rate without loss of glycol yield. These authors describe several recovery methods, one a countercurrent extraction with butanol, the other a flash distillation with kerosene vapor.

The staff of the Northern Regional Research Laboratory carried through a number of experiments on fermentation aspects of 2,3-butylene

glycol production which were reported in part by Ward, Pettijohn, and Coghill (141). Since recovery of the glycol was a difficult problem, these workers investigated the use of acid-hydrolyzed, refined wheat and corn starches fermented by *Aerobacter aerogenes*. Their object was to develop the best cooking and fermentation conditions for the organism in this simplified medium. Small-scale experiments were run in rotating aluminum drums. The medium was prepared by hydrolysis of the starches at 22–25 lb. for periods up to four hours. Hydrochloric and sulfuric acids in concentrations from 0.02 to 0.12 *N* were used to hydrolyze the starch. The hydrolyzed starch with mineral salts, urea, and calcium carbonate made up the medium. It was found that the trace element content of the mash was a critical matter and this was attributed to the use of a purified raw material. Whole grain mash was not affected by trace element additions. When the mash was supplemented in a manner suited to the particular starch under investigation, a yield of 13.5 to 14.0 lb. of 2,3-butanediol was obtained from 34 lb. of starch (equivalent to one bushel of corn). Ion exchange resins were used with benefit in some cases as a means of correcting unbalance in the trace element content of the mash.

## 2. Research on *Bacillus polymyxa*

Comprehensive investigations were carried out on *B. polymyxa* fermentation of starch by a group of investigators in Canada, where a surplus of wheat furnished potential raw material for this type of fermentation. The results of this work, which was done under sponsorship of the National Research Council of Canada, have been published (1–4,42,66,67,73,91,107, 123). A general preliminary investigation by Ledingham, Adams, and Stanier (73) showed that there was considerable variation between strains of *B. polymyxa* and also that individual strains of the organism showed a marked tendency to dissociate with resultant changes in fermentative capacity. The authors worked out general conditions for the fermentation. It was found that whole wheat could be fermented at concentrations up to 15%, that 32.5°C. was the optimum temperature, and that yeast extract was a beneficial supplement. The fermentation time was sixty hours and the glycol-ethanol ratio was of the order of 1.3 to 1.0, under anaerobic conditions. The same authors (123) reported on the filterability of mash fermented by *B. polymyxa* since filtration was considered to be a necessary step in processing. Filterability varied with strains and was not correlated significantly with yield. Evaluation of a strain requires determination of both factors. The authors recommend lyophilizing cultures in order to

stabilize them. Adams and Stanier (4) reported on a biochemical study of the fermentation. Carbon balances were made on glucose fermentations with good recovery of carbon. Xylose, pyruvic acid, and mannitol were fermented and the variations in products determined. Depending on the substrate large differences occur in the glycol-ethanol ratio and there are differences in acid production as well. Attention is drawn to the fact that the organisms producing butylene glycol are widely separated taxonomically, but are similar in their metabolism. Neish (91) continued the studies on *B. polymyxa* by investigating the optical activity of the glycol produced from fermentation of wheat. He showed that only the *l*-form was present.

Rose and King (107) operated a small scale production and recovery unit. This consisted essentially of: (1) a cooking vessel, (2) fermenters, (3) a filter, and (4) a still pot with packed column for glycol recovery. The still was operated under vacuum. The entire set-up was simple in design and involved fermenting only 5 gal. of mash. But the data obtained could presumably be applied to larger scale operations. For instance, it was found necessary to malt the mash under conditions such that it was well liquefied. This prevented the formation of lumps with accompanying infection from butyric acid organisms. Filtration of the mash following completion of the fermentation was very difficult and the problems connected with this step were not solved completely. Distillation was carried out successfully by passing steam through filtered beer in the still pot and fractionating by means of the packed column. The first distillate required purification by several redistillations and chemical treatments. Over-all recovery including the first distillation was 55% of the glycol in the beer.

In the *aerogenes* fermentation of sugars, aeration and mechanical agitation are both necessary for maximum glycol production. Adams (1) studied the relationship of aerobic and anaerobic conditions to the formation of glycol in the *B. polymyxa*-type of fermentation. Aerobic conditions were obtained by use of the now well-established method of shaking the flasks containing the inoculated culture medium. Generally speaking aeration by shaking decreased both glycol and ethanol formation. Shaking toward the end of the fermentation period only had a slightly beneficial effect in raising both yield and the glycol-ethanol ratio. This paper includes an interesting study of the effect of passage of various gases through the fermenting solution. Air and oxygen increased rate of formation and yield of glycol, decreased that of ethanol. Passage of nitrogen or hydrogen had a similar effect and in addition the fermentation time was shortened.

This suggests that sweeping out the fermentation carbon dioxide is important for maximum glycol production, as is claimed in the patent granted to Scheffer (114). This is further supported by the work of Adams and Leslie (2) on the surface-volume effect in these fermentations and the effect of reduced pressure.

Adams and Leslie (3) made a study of the maintenance of optimum pH in mashes fermented with *B. polymyxa*. Control of this factor is necessary because the organism produces acids which will ultimately stop the fermentation. Acidity may be controlled by addition of calcium carbonate but ammonia would be more desirable as a neutralizer in an industrial process because it is easily handled and does not add to the ash in the residue and thus lessen its feed value. The authors showed the use of ammonia to be practicable. Fratkin and Adams (42) studied the use of wheat from which some of the gluten and other fractions of the grain had been removed to give a crude wheat starch. This material was considered because its use would lessen processing difficulties. The addition of malt sprouts was found to be the most effective fermentation supplement. With these added, the crude starch could be fermented with good yields in about 72 hours.

The tendency to variation in *B. polymyxa* which is brought out by the above studies makes it unsuited to commercial use. In addition it produces considerable ethyl alcohol, the fermented mash is difficult to filter and process, and the total concentration of products is less than in the *aerogenes* fermentation. Experimenters have thus come to the conclusion that if grain is to be used, it would be preferable to acid-hydrolyze the starch to sugar and ferment with *aerogenes*. This is further supported by the work of Katznelson (66,67), who showed *B. polymyxa* to be quite sensitive to bacteriophage.

## C. PROBLEM OF RECOVERY OF GLYCOL FROM FERMENTED MASH

### 1. Steam Stripping

One possible method for the recovery of 2,3-butyleneglycol from fermenter liquors is by steam stripping in a suitable column. This required study on a pilot plant scale in order to prove its practicability. The raw materials for the fermentation might be either molasses or grain. In either case, following the fermentation of the sugar, it would be necessary to separate the glycol from a relatively large amount of dissolved and suspended material. In the fermentation solution from the grain process, glycol would be present in a concentration of about 3.5 to 4.0%, acetyl-

methylcarbinol in a concentration of 0.1 to 0.2%, and ethanol in a concentration of 0.3 to 0.5%. Blom, Reed, Effron, and Mustakas (18) studied the stripping of the glycol from this mixture by means of steam under pressure. As a preliminary, the fermentation liquor was concentrated in an evaporator so that it contained about 13% glycol. The alcohol was recovered from this preliminary evaporation as a condensate from certain evaporator effects. Glycol is a relatively high-boiling liquid but considerable loss in the vapor would occur in the ordinary evaporator, and to prevent this it is necessary to have rectifying columns on the effects. In the experiments of Blom and co-workers the syrup from the evaporator was pumped to the top of a packed column, through which it flowed counter-current to steam at 55 lb. pressure. This pressure stripping was adopted for purposes of steam economy since it gives a favorable ratio of butylene glycol in the vapor to that in the liquid.

The authors ran the stripping process for as long as seven days in order to obtain the operating data on capacity, cycle of feeding, cleaning and caustic wash, and other factors necessary to evaluate this process for obtaining glycol. Their paper gives a plant flow sheet for the handling of beer from butylene glycol fermentations of acid-hydrolyzed wheat mash. This includes a quadruple effect evaporator equipped with rectifying columns for the vapor from the various effects, a stripping column to remove the glycol, and a scrubbing column for the final purification of the glycol vapors.

They conclude that this method of recovery appears to be feasible both mechanically and from the point of view of economy of steam, since the vapors from the pressure-stripping operation can be used after passing through the scrubbing column as process steam elsewhere in the plant.

### *2. Solvent Extraction as a Recovery Method*

Liquid-liquid extraction as an alternative to recovery by steam stripping was studied by Othmer, Bergen, Schlechter, and Bruins (96). A number of solvents were considered and ternary diagrams were made for certain solvents. Operating temperatures were investigated. Flow sheets for preliminary treatment of the liquor and for solvent extraction were suggested on the basis of the laboratory work. As a contribution to information on the general process, Othmer, Schlechter, and Kozalka (97) studied the composition of vapors from boiling binary solutions using liquids involved in solvent recovery, acetylation, and other process steps.

#### D. ACETYLATION OF GLYCOL

Acetylation of the glycol was necessary in order to provide the material for the pyrolysis step yielding butadiene. A number of investigators had

tried direct catalytic dehydration of the glycol to butadiene but results had been invariably disappointing. Yields were usually of the order of 10% and never above 20%. Schniepp, Dunning, and Lathrop (116) undertook an investigation of acetylation as a means of obtaining butadiene from glycol. The work was carried through laboratory stages and batch preparations. It was realized that large-scale operations would require continuous processing and these workers laid the groundwork for such a process. Their report includes a flow sheet for a plant to carry out the continuous acetylation of glycol based on pilot experiments.

In developing a satisfactory continuous process, certain conditions had to be met in order for the scheme to succeed. The most important of these were: (1) the acetylation had to go to completion; (2) time in the acetylation apparatus had to be kept low; (3) by-products were to be avoided; and (4) unreacted acetic acid had to be recovered completely to avoid a high processing charge against the produce due to loss of this reagent. Schniepp, Dunning, and Lathrop devised a procedure for meeting the above conditions. It was found that complete conversion in a reasonably short time depended on prompt removal of the water produced by the esterification reaction. This could be accomplished by using an entrainer which was in this case excess acetic acid in the system. The formation of by-products was troublesome at first. Some monoacetate was formed, as well as butadiene in small amounts, methyl ethyl ketone, and a small amount of tar. These difficulties were avoided by countercurrent operation of the acetylation column and careful temperature control. As finally developed, the process involved feeding the glycol with sulfuric acid as catalyst into the top of the reaction column and at the same time feeding glacial acetic into the base of the column. The distillate contained acetic acid, water, and traces of methyl ethyl ketone; the base product consisted of glycol diacetate, acetic acid, and sulfuric esters of glycol. Both of these were further processed, the distillate to give glacial acetic acid for reuse in esterification and the base product to give the desired diacetate. It was found possible to produce 97% diacetate from the input glycol. The system is said to be applicable to production of other high-boiling acetic acid esters.

Shlechter, Othmer, and Marshak (121) report laboratory studies on the rates of esterification of glycol. Their work throws some light on the nature of the reaction. They also give equilibrium data which would be helpful in designing large-scale equipment for the process.

## E. PYROLYSIS OF GLYCOL DIACETATE

The final step in the production of butadiene from fermentation glycol is pyrolysis of the diacetate. Morell, Geller, and Lathrop (89) undertook a laboratory study of the conditions necessary for this part of the process. They investigated temperature, contact time, number of passes required, by-products of the reaction, and acetic acid recoveries. Before working with the diacetate, attempts were made to dehydrate the glycol directly by catalytic means. Some seventy catalysts were tried under a variety of conditions but the highest yield obtained was 20% butadiene in one pass, with the main product methyl ethyl ketone. This agrees with the results obtained by other investigators. The work of Morell, Geller, and Lathrop made possible the engineering of a pilot plant. Yields of 82% butadiene of 99% purity were obtained in one pass between 575° and 600°C. Acetic acid was not broken down since it could be recovered quantitatively either as such or in combination with by-products. A study of the pyrolysis step on a laboratory scale was also made by Schlechter, Othmer, and Brand (120). Their results are in substantial agreement with those obtained by Morell and co-workers. They recommend carrying out the pyrolysis reaction in an atmosphere of nitrogen. By diluting the diacetate in the vapors to about forty moles per cent with this gas, it was claimed that polymerization during pyrolysis was reduced.

On the basis of laboratory work of Morell and co-workers, a pilot plant was set up for further study of the pyrolysis step. Results are reported by Schniepp, Dunning, Geller, Morell, and Lathrop (115). The equipment consisted essentially of a metal pyrolysis coil in a bath of molten lead for the main step of the process. Vapors from the coil were cooled rapidly in a quench chamber and passed through a packed column where acetic acid and other liquid pyrolysis products were washed out. The butadiene gas was scrubbed, dried, compressed, and finally condensed to liquid for collection and weighing. It is interesting at this point to note the pilot plant recoveries tabulated by Schniepp and co-workers. These are given below as over-all recoveries of butadiene from the glycol.

Materials charged	Pounds	Products and recoveries	Pounds
2,3-Butylene glycol..	195.16	1,3-Butadiene.....	100.0
Acetic acid.....	519.84	Acetic acid.....	517.39
Sulfuric acid.....	3.46	Methyl ethyl ketone.....	17.54
		Hydrocarbons and tars.....	2.20
		2,3-Butylene glycol diacetate..	1.83
		Noncondensable gases.....	2.87

There was thus recovered 0.512 part of butadiene per part of glycol introduced into the esterification-pyrolysis process. Had it been necessary, the manufacture of butadiene could have been carried out by the fermentation of sugars to 2,3-butylene glycol, recovery of this glycol, its subsequent esterification to the diacetate, and the pyrolysis of the diacetate to give the desired synthetic rubber raw material. This was not necessary since other raw materials served the purpose. If the processes noted above had been put into large-scale operation, considerable plant construction would have been required. In view of wartime shortage of equipment it is fortunate that this was not essential. However, the knowledge gained by these studies will gradually find application either in fermentation projects or otherwise, which justifies the effort put into their development.

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